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Characterization of intracellular survival of Mycobacterium avium subspecies paratuberculosis in J774 murine macrophages

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Characterization of intracellular survival of *Mycobacterium avium* subspecies *paratuberculosis* in J774 murine macrophages

By

Jesse Michael Hostetter

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For the Graduate College

This dissertation is dedicated to:

Mary S. Hostetter, MD

John I. Hostetter, MD

TABLE OF CONTENTS

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GENERAL INTRODUCTION

Statement of The Problem

Mycobacterium avium Subspecies *paratuberculosis* (*M. a. ptb*): Intracellular Pathogens of Macrophages

M. a. ptb is the causative agent of Johne's disease (see appendix A) and, like other pathogenic species of mycobacteria, is an intracellular pathogen of monocytes and monocyte-derived macrophages (20). Macrophages are phagocytic cells which play a key role in both innate and acquired defenses. Macrophages identify, ingest, and eventually kill infectious agents through generation of several toxic substances including oxygen derived free radicals, nitric oxide, acid hydrolases, and proteolytic enzymes (4). Macrophages also process antigens and present them to T-cells leading to development of an adaptive immune response (15). Several pathogens (bacteria, fungi, viruses) have developed strategies which enable them to survive and replicate within the macrophages. *M. a. ptb* is one such pathogen; however, the mechanisms by which it survives within macrophages have not been well-characterized. Determining how pathogenic mycobacteria avoid the intracellular destructive processes and remain sequestered from the immune system is an area of important research interest.

Numerous advances have been made using cultured macrophages to evaluate intracellular survival strategies of several species of mycobacteria including *Mycobacterium tuberculosis* and *M. avium*(3, 7, 9, 11). These studies have demonstrated that these species of pathogenic mycobacteria reside within an intracellular vesicle (phagosome) that does not follow the normal maturation steps, instead these phagosomes form a protective environment for the bacteria. Pathogenic mycobacteria reside and control the environment

within this phagosome, and remain sequestered from the degradative environment of the phagolysosome. The characteristics of the mycobacterial vacuole are summarized below.

Phagocytosis

Mycobacterium tuberculosis and *M. avium* have developed mechanisms which manipulate uptake and influence the response generated by the macrophage following phagocytosis. Macrophages recognize and bind particles through specific and nonspecific surface receptors. Certain receptors such as the complement receptors have been shown to have limited ability to stimulate intracytoplasmic killing mechanisms following their ligation (1, 19). One strategy used by both *M. tuberculosis* and *M. avium* is to initiate uptake by binding to these receptors, thereby inhibiting certain antimicrobial mechanisms including generation of reactive oxygen intermediates(16, 17).

Inhibition of Phagosomal Acidification

Mycobacterium tuberculosis and M. avium reside within a phagosomal compartment which fails to become acidified(5, 13). Progressive phagosomal acidification normally begins shortly after internalization(5, 13, 14, 18). The resulting acidic environment within the phagosome is an important antimicrobial response, which is directly toxic to many types of microorganisms, and is required for optimum activity of hydrolytic enzymes including the acid protease cathepsin D (10, 18). Therefore the ability to interfere with phagosomal acidification is an important virulence factor for mycobacteria.

Inhibition of Phagolysosome Formation

The phagosomes containing pathogenic mycobacteria continue to communicate with early endosomes, but do not mature into phagolysosomes(2, 6). Phagosomal compartments containing ingested particles communicate with all stages of the endosomal system with

subsequent intermingling of membrane components and vesicle contents. It is through sequential interactions with early, late, and lysosomal compartments that the phagosome matures into a phagolysosome (8, 12). Phagosomes containing *M. avium* and *M. tuberculosis* remain in contact with early endosomal compartments, but do not interact with late lysosomal compartments. It is by this selective fusion with only early endocytic compartments that phagosomes containing *M. tuberculosis* and *M. avium* resist maturation into phagolysosomes(7, 18).

Specific Aims

It was our intention to determine if the strategies employed by these pathogenic species of mycobacteria were also involved in the intracellular survival of *M. a. ptb*. We accomplished this goal with a series of *in vitro* assays using the murine macrophage cell line J774. It was our hypothesis that *M. a. ptb* survives intracellularly by inhibiting maturation of the surrounding phagosome into a functional phagolysosome. This hypothesis was tested by meeting the following objectives: 1) characterize the pH of the phagosome containing *M. a. ptb* (Chapter 2) ; 2) identify stage specific markers on the phagosome containing *M. a. ptb* to determine its degree of maturation (Chapter 3); and 3) characterize intracellular survival of *M. a. ptb* in cytokine treated macrophages (Chapter 4).

Dissertation Organization

This dissertation is organized in the alternative format. This dissertation is composed of a general introduction and literature review (chapter 1) which is followed by the three manuscripts (chapters 2-4) that have been prepared for submission to the journal Veterinary

Pathology, followed by general conclusions. References are cited at the end of each chapter. One appendix is included which is brief overview of paratuberculosis.

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CHAPTER ONE: LITERATURE REVIEW

Phagosomal/Endosomal Trafficking

Phagocytosis

Phagocytosis, pinocytosis, and receptor-mediated endocytosis are mechanisms that lead to internalizing of extracellular material by animal cells. Pinocytosis is the mechanism used for the uptake of fluids and solutes, while closely related-receptor mediated endocytosis is involved in uptake of macromolecules and small particles (1). Phagocytosis is the process whereby larger material such as microorganisms and other particles greater than .5μm are internalized; it serves as a vital defense mechanism for the host. Most cells within the body are capable of some phagocytosis. However, macrophages, monocytes, and neutrophils are highly efficient at phagocytosis (1). Following phagocytosis by these cells, the engulfed microorganism is exposed to a progressively more hostile environment which includes a low pH, hydrolases, and reactive oxygen intermediates generated during the oxidative burst (93). Phagocytosis eventually leads to killing and degradation of the ingested material with processing and presentation of antigenic peptides (97). Phagocytes are important components of innate immunity and following antigen processing (by macrophages) are also key mediators of adaptive immunity (62, 97).

Phagocytosis is initiated by recognition of the particle or microorganism through receptors on the cell surface. Many receptors specifically bind particles and initiate phagocytosis and they have been grouped into two basic categories. The first are those receptors that interact indirectly with the particle by binding to material derived by the host, forming a bridge between the particle and the phagocyte (88). These bridges are called opsonins and the process is called opsonin-dependent phagocytosis. Invading organisms such

as bacteria can be opsonized nonspecifically by complement proteins which are present in serum, or may be opsonized by immunoglobulin generated in an immune response (4). Fc gamma (Fcγ) and complement receptors (CR), namely CR3, are transmembrane glycoproteins which bind opsonized particles and are the two main classes of receptors described for opsonin-dependent phagocytosis (128). Generally Fcγ receptors are present on the surface of macrophages and lead to phagocytosis after binding to their ligand; however, the CR3 requires further cell activation before phagocytosis will progress(14, 73). The second class of receptors are those that bind directly to a structural component of the particle and this process is named nonopsonin-dependent phagocytosis (125). This form of phagocytosis provides the host with an alternative mechanism for recognizing invading microorganisms in the absence of antibody or complement (88). There are numerous nonopsonic dependent receptors including the mannose receptor, endotoxin receptors, β1 and β3 integrin receptors, and scavenger receptors(1).

Binding of a particle to a given receptor leads to polymerization of actin with subsequent internalization. However, the cellular mechanisms that lead to these events are variable and are dependent on the type of receptor. As described by the "zipper hypothesis" actin polymerization occurs at the tip of the pseudopod and progresses around the particle with sequential binding of receptors to the particle surface leading to a portion of the cell membrane surrounding the particle(53, 121). This is followed by invagination into the interior of the cell and budding off of the membrane from the surface to form the phagosome (125). Different morphologic features of uptake have been identified that vary according to the type of receptor engaged. Complement-opsonized particles tend to be directly internalized into macrophages after binding to their receptor, whereas the membrane

protrudes from the cell surface to tightly surround bound IgG opsonized particles prior to their uptake (73). The signal transduction pathways leading to actin polymerization are also variable and are dependent on the type of receptor engaged. During CR mediated phagocytosis protein kinase C is required for actin polymerization; however, tyrosine kinases do not appear to be involved. In contrast, tyrosine kinase is an absolute requirement for actin polymerization following binding to the FcγR (4). Engagement of different types of receptors also leads to variation in the cytoskeletal rearrangements occurring during phagocytosis. Phagocytosis of complement or IgG opsonized particles results in a phagosome which recruits the cytoskeletal proteins vinculin and paxillin, however these proteins are not recruited to the phagosome derived from nonopsonic dependent phagocytosis (4).

In addition to variation in cell signaling and cytoskeletal rearrangement, the ability of the cell to produce pro-inflammatory mediators during ingestion is dependent on the receptor used for uptake of the particle. Phagocytosis via the FcγR is closely associated with production of reactive oxygen intermediates including H_2O_2, and metabolites of the arachidonic acid cascade (128). Similar to the FcγR, phagocytosis via the mannose receptor tends to be pro-inflammatory with several extracellular signaling factors including IL-12 being produced following ligation (109). In contrast, H_2O_2, metabolites of the arachidonic acid cascade, and other mediators of inflammation are not produced following phagocytosis by complement receptors, and this may represent a mechanism for the clearance of complement opsonized particles without an inflammatory response(2, 136).

Phagosomal Maturation

Phagosomal and endosomal pathways

The phagocytic and endocytic pathways are intimately related. The endocytic pathway leads to uptake of solutes, membrane compounds, and ligands eventually leading to their recycling or accumulation within lysosomes. Materials that are taken in by the cell progress through a pathway that has been divided into four sets of organelles (57). The first is the *early endosome*, which is formed immediately following uptake. Some materials, including certain receptors, will not be degraded but instead will be selectively retrieved from this compartment and recycled to the cell surface (recycling endosomes). The second is the *endocytic carrier vesicle*, which is also known as the multivesicular body. The third organelle is the *late endosome* that will deliver contents to the last of the organelles of the endocytic pathway, the lysosome. Like the early endosome, contents of the late endosome, namely the mannose-6-phosphate receptor, are also recycled. Here recycling is mainly to the trans-Golgi network, however there is some evidence that there is also recycling to the plasma membrane (98). Lysosomes are the endpoint of the endocytic and phagocytic pathways and serve as the major degradation site for proteins and lipids within the cell (114).

Progression through the endocytic pathway involves a complex and incompletely understood series of events leading to lysosome formation. Two main hypotheses, the "maturation", and the "vesicular shuttle", models have been proposed to describe how materials pass along these organelles. The maturation model states that endocytic vesicles are transformed as they progress through the pathway and eventually mature into lysosomes (85). In the "vesicle shuttle model" transport vesicles connect pre-existing endosomal compartments (54). In both of these models there are fusion and fission events occurring

between endocytic organelles and the true mechanism may actually be a combination of the two models (124). A third model has also been described concerning the formation of lysosomes and is known as the "kiss and run" model. In this model lysosomes are generated by repeated series of transient fusion and fission processes with late endosomal compartments (114).

Following phagocytosis there are numerous interactions between the phagosome and endocytic organelles which lead to phagolysosome formation (80). Immediately following phagocytosis, the membrane surrounding the phagosome is identical to that of the cell membrane, while the phagolysosome, which is the endpoint in this pathway, has nearly all of the traits of a very large lysosome(84, 114). The mechanisms involved in phagolysosome development are also complex and incompletely understood, however phagosomes are known to move through the cell along microtubules and mature in a process thought to be similar to that of endosomes to lysosomes (15, 92). Interaction of the phagosome with endosomes and lysosomes is required for phagolysosome formation and phagosomes have been shown to fuse *in vitro* with all organelles of the endocytic pathway, and the specificity of fusion with endosomes tends to vary with age of the phagosome (70, 80).

These fusion events allow for numerous phagosomal modifications to occur including alteration of the phagosomal membrane components, and acquisition of endosomal proteins. Initially phagosomes tend to fuse with early endosomes, mixing membrane components and contents. Subsequently the phagosome will become unable to fuse with early endosomes and will then fuse and intermingle with late endosomes, and eventually fuse only with lysosomes (phagolysosome formation)(33, 37). As a result, as phagosomes mature they obtain functions initially of early endosomes, including the ability to recycle proteins to the cell surface, then

acquire degradation functions of late endosomes and lysosomes(92, 93). Although membrane components of phagosomes and endosomes are very similar there are some important differences. Within the phagosomal membrane is an electron transport chain which accepts electrons from NADPH in the cytosol to reduce oxygen producing superoxide within the phagosome and this electron transport chain has not been identified within the membranes of endosomes(92, 108).

It has been hypothesized that the communication between endosomal organelles and phagosomes is not complete fusion, but instead consists of exchange of fluid and membrane components following a brief fusion event followed by fission as described in the "kiss and run" interaction (36, 114). This hypothesis is supported by demonstration of such transient fusion events between endosomes and phagosomes containing various endocytic and phagocytic tracer compounds(36, 37).

Markers of phagosome/endosome maturation

Interaction between the phagosome and endocytic organelles during phagosomal maturation results in acquisition and loss of numerous stage specific proteins from the phagosomal membrane (84). Over 200 different polypeptides have been isolated from early and late endosomes, and a few, which have been well-described, serve as membrane markers characterizing phagosome and endosomal maturation (35). The presence or absence of these markers can be used to classify these compartments as early, late, or lysosomal in nature. The types of proteins described within endosomal/phagosomal compartments and membranes include structural, enzymatic, and receptors, and several with as yet unknown function. Detailed description of all of these markers is beyond the scope of this manuscript, however some that are well characterized (listed in Table 1) are discussed in the paragraphs below.

Table 1.

Markers of phagosome/endosome maturation

Early Markers	Late Markers
Transferrin Receptor	Lamp-1
Adaptins	CD63
Rab 5	Cathepsin D
	Rab7

Many of the markers of early endosomes and phagosomes are proteins associated with the plasma membrane. These include several types of receptors present at the cell surface such as the transferrin receptor, the mannose receptor, and the Fc II receptors (23). Following uptake, these receptors are selectively removed from their vesicles and recycled to the cell surface resulting in their early loss during phagosome/endosome maturation (93). The transferrin receptor is a typical example of an early endosomal marker. These receptors are synthesized in the endoplasmic reticulum and following passage through the Golgi complex are transported to the cell surface. At the surface they bind iron bound transferrin and are subsequently internalized into coated vesicles that fuse early endosomes. Acidification of this endosomal compartment releases the iron from transferrin and the unsaturated transferrin and the receptor are recycled to the cell surface. Transferrin and its receptor are specific for the early endosomes and do not traffic through the lysosomal compartment(29, 74). Adaptins are peripheral membrane proteins linked to clathrin lattices to the plasma and trans-Golgi membranes(18, 59). Clathrin lattices have been demonstrated on developing phagosomes, but are rapidly lost following completion of phagocytosis, and

adaptin proteins changes in the phagosomal membrane parallel that of the clathrin lattices(3, 93).

Markers of late phagosomal stages are acquired from interaction with the late endosomal network and become concentrated within phagosomes at late stages in their development. One of the best-characterized late markers is the lysosome-associated membrane protein 1 (Lamp1). This is a highly glycosylated membrane protein with a short C-terminal domain, a single transmembrane domain, and a large intraluminal domain. Lamp concentration increases within the phagosome during maturation, and Lamp-1 is enriched in late phagosomes and phagolysosomes (114). The function of Lamp 1 is currently unknown, however it may play a role in resistance to membrane degradation to hydrolases and other destructive enzymes (45). Cathepsin D is a proteolytic enzyme found within lysosomes and is a well-characterized marker of late phagosomal stages. Procathepsin D, which is the proenzyme form of cathepsin D, is synthesized within the trans-Golgi system and is delivered to the early components of the endosomal system. The proenzyme is sequentially cleaved during maturation to yield the active forms within late phagosomes and phagolysosomes(66, 99). CD63 is a glycoprotein that was originally characterized as a marker of platelet activation, however it has also been identified in several other cell types. In macrophages, CD63 has been shown by immunoelectron microscopy to colocalize intracellularly with Lamp-1 and has since been used as a marker of phagolysosomes (83).

Rab proteins

Proteins belonging to the Rab family have been identified as markers of endosome/phagosome maturation, and have been shown to have an important role in trafficking of vesicles of the endosomal and phagosomal networks. Rab proteins are Ras-

related small molecular weight GTP binding proteins which have regulatory functions associated with docking and fusion of membrane bound vesicles within a cell (51). Like all small GTP binding proteins, the Rabs are in their active form when they are bound to GTP and are in their inactive form when bound to GDP. When in the active form these proteins allow fusion of vesicles containing homologous GTP binding proteins (25). Two of these proteins, Rab5 and Rab 7, are closely associated with endosomal/phagosomal maturation, but with distinct functions. Rab5 is present both on early endosomes/phagosomes and the cytoplasmic face of the plasma membrane, and is required for early endosome/phagosome fusion (51). In contrast Rab7 is localized on late endosomes/phagosomes and functions downstream of Rab5 in membrane transport to late endosomes/phagosomes (40).

Rab proteins are part of a highly regulated, multiple protein complex which facilitates docking and fusion between vesicle and target membranes. Members of this complex include the general fusion proteins N-ethylmaleimide-sensitive protein (NSF), soluble NSF attachment proteins (SNAPs). Additional members are the SNAP receptors (SNAREs), which are present both on the vesicular membrane(v-SNARE), and on the target membrane (t-SNARE) (111). The mechanisms leading to fusion are incompletely understood. It appears that during interaction with NSF/SNAP the v and t-SNAREs on apposing membranes fuse to form a complex called a SNAREpin. This SNAREpin then allows for fusion of the target and vesicle membranes(111, 134). The role of the Rab proteins in membrane fusion is unclear and there may be differing mechanisms used by different members of the Rab family. In yeast it has been demonstrated that when Rab proteins are activated they function by displacing inhibitory proteins allowing interaction of the v-SNAREs and t-SNAREs (77).

Additionally, Rab5 has been shown to regulate early endosome interaction by stimulating early endosomal association and motility along microtubules in vitro (86).

Factors that influence phagosomal maturation

Phagosomal maturation is not a uniform process for all particles and varies according to the type of particle ingested. Particles such as killed bacteria that are easily degradable tend to be processed efficiently, and phagosomes that have taken up killed bacteria mature into phagolysosomes. In contrast, particles that resist degradation, such as some types of latex beads, tend to be within phagosomes that continue to fuse with early endosomes, and do not progress to phagolysosomes (31).

Phagolysosome formation is also influenced by surface properties and size of the particle. Particles that are less than 0.5 μm are processed normally to phagolysosomes. Particles greater than 1.0 μm with hydrophobic surface properties were present in phagosomes that did not mature to phagolysosomes, while those with hydrophilic surfaces did (33). Differences in processing of these particles may be related to the interaction of the particle surface and the phagosomal membrane resulting in differences in the tightness of the apposition between these surfaces. The hydrophobic particles (greater than 1.0 μm) result in a tightly apposed membrane which may interfere with phagosomal interaction with the endosomal organelles and inhibit maturation of the phagosome (33).

Particle degradation and processing

Phagolysosome formation leads to degradation of the ingested particle, and fragments of the particle are then processed for presentation to T cells. The acidic environment within the phagolysosome causes activation of numerous proenzymes including cysteine proteases and acid hydrolases, which are delivered to the lysosomes and phagolysosome from the

trans-Golgi network. Newly synthesized degradation enzymes are sorted to lysosomes and phagolysosomes via vesicles which are tagged with the mannose-6-phosphate receptor, which is recycled back to the Golgi prior to delivery of contents to lysosomes/phagolysosomes(55). Class II MHC molecules have been demonstrated within both lysosomes and phagolysosomes and these organelles have been shown to be capable of antigen processing leading to formation of antigen-MHCII complexes(62, 97). In addition to degradation within phagolysosomes, phagocytosed material has also been shown to be degraded and processed in late endocytic organelles and lysosomes following exchange of phagosomal contents via phagosome-endosome interaction (126). Following their formation, antigen MHCII complexes are transported from the phagolysosome and lysosomal compartments to the cell surface via endosomal recycling vesicles(62, 137).

Phagosomal acidification

After particle ingestion and phagosome formation, the pH within the phagosomal compartment progressively decreases, and the resulting acidic environment is important for generation of antimicrobial activity within the phagocyte. The phagosomal pH rapidly drops to below 5.5, which is toxic to many types of microorganisms(60, 118). In addition to being directly toxic, low phagosomal pH is also important in activation of other degradative functions. Many of the hydrolytic enzymes which are secreted into lumen of the phagosome are optimally functional at a low pH (60). Low pH also enhances production of reactive oxygen intermediates by increasing hydrogen peroxide generation, which also serves as a substrate for myeloperoxidase(44, 61). Acidification of the phagosome is also associated with phagosomal maturation and appears to be a requirement for phagolysosome formation(93, 115). In contrast to the dependence of phagosomal processing on type of

receptor used, phagosomal acidification is an independent process from the mode used for uptake of the particle (16).

Although a number of mechanisms have been proposed for reducing phagosomal pH, acidification is most likely achieved via translocation of H^+ from the cytosol to the phagosomal space. A number of candidates that may contribute to phagosomal acidification have been identified including the vacuolar-type H^+-ATPase (V-ATPase), Na^+/H^+ exchanger (NHE), Na^+/K^+-ATPase, and the chloride channel (118). The V-ATPase proton pump has been shown to be the major contributor to phagosomal acidification by demonstrating that phagosomal acidification is inhibited by the selective V-ATPase inhibitor bafilomycin $A_{1(76)}$. These findings are further supported by evidence that neither the Na^+/K^+-ATPase nor NHE contribute significantly to proton accumulation within phagosomes (60).

V-ATPase proton pump

The V-ATPase is a multimeric protein complex found in most eukaryotic cells and is responsible for acidification of intracellular compartments and maintenance of cytoplasmic pH. V-ATPase is a member of a family of ATP dependent proton pumps. V-ATPases are structurally related to F-ATPase, which maintains the proton motive force across the inner mitochondrial membrane, and it is likely these two enzymes were derived from a common genetic ancestor (17). Based on its structural similarity to the F-ATPase, the V-ATPase is thought to function by a similar mechanism, however instead of generating ATP as F-ATPase does, ATP is hydrolyzed with the resultant energy expended on pumping hydrogen ions across cell membranes generating an H^+ ion gradient (52).The V-ATPase is a transmembrane protein that has two functional domains the V_1 and V_0. The V_1 is the peripheral domain which functions in ATP hydrolysis and the V_0 which is the membrane

spanning domain is involved in proton translocation (8). V-ATPase, like other proteins delivered to the phagosome during maturation, appears to be acquired from interaction with the endosomal network. Increasing concentrations of V-ATPase have been detected on phagosomal membrane within a short time of internalization(93, 118). This coincides with evidence that acidification is an early event following phagosome formation preceding phagolysosome formation (81). In addition to phagosomal membranes, V-ATPases have been demonstrated within the plasma membrane of murine macrophages where they function by extruding hydrogen ions from the cell, maintaining the cytoplasmic pH within narrow limits(119, 120).

Survival of Intracellular Pathogens

Macrophages play a vital role in host defense by ingesting and killing invading microorganisms and by subsequently processing and presenting antigen to T-cells for generation of an adaptive response. Several types of pathogens (including a number of species of bacteria, fungi, protozoa, and viruses) are capable of intracellular survival and replication following their uptake. A number of different strategies have emerged which allow these organisms to circumvent the killing processes generated following phagocytosis and phagosome formation.

Although there is some variation from one type of pathogen to the next, four main strategies have been described that allow intracellular pathogens to bypass killing mechanisms (125). *Listeria monocytogenes* is an example of the first type of strategy, which is escape into the cytoplasm. Following uptake, listeria initially resides within the phagosome, but following secretion of two bacterial enzymes, hemolysin and phospholipase C, the phagosomal membrane is lysed and the bacteria escape into the cytoplasm(19, 95).

Leishmania mexicana is an example of the second strategy which is survival within the phagolysosome. Leishmania remains within the phagosome and eventually the phagolysosome, and here is capable of survival by resisting degradation by lysosomal hydrolytic enzymes (103). In addition to withstanding enzymatic assault, Leishmania is also able to withstand the toxic affects of acidification as the pH of the *Leishmania amazonensis* phagosome has been demonstrated to ranges from 4.7 to 5.2 in established infections (6). *Yersinia pseudotuberculosis* is capable of intracellular survival by the third strategy, which is inhibition of phagosomal acidification. Yersinia decrease acidification by inhibition of V-ATPase proton pump activity (127). *Legionella pneumophila* is an example of the fourth and final strategy of intracellular survival, which is creation and control of an environment within a bacteriophorous vacuole . *Legionella* survives by avoiding the destructive processes of the phagolysosome by residing in a phagosomal compartment that does not mature into a phagolysosome (6). Although these strategies have been well described and numerous examples exist for each category, it is likely that a given pathogen uses more than one for successful intracellular survival. *Listeria monocytogenes* is one such pathogen. *Listeria's* escape from the phagosomal compartment is well described, however evidence also indicates that *Listeria*, like *Legionella*, is capable of inhibiting maturation of its phagosome (5). *Legionella pneumophila* is another example of employment of multiple strategies. In addition to inhibition of phagosome maturation, *Legionella*, like *Yersinia*, inhibits phagosomal acidification (68).

The Mycobacterial Phagosome

Introduction

Several species of mycobacteria cause disease by possessing the ability to reside and replicate within the host macrophage. Here they avoid numerous intracellular killing mechanisms and remain sequestered from the host's immune system. Like many intracellular pathogens, pathogenic species of mycobacteria use multiple strategies to survive within macrophages, namely inhibition of phagosomal acidification and inhibition of phagosome maturation. Knowledge of the mechanisms used by pathogenic species of mycobacteria to inhibit phagolysosome development comes from a large body of research concerning intracellular survival of two pathogenic species *M. tuberculosis* and *M. avium*in both the human and veterinary medical literature. The remainder of this section will be dedicated to a review of the survival mechanisms characterized in these and related pathogenic mycobacteria.

Attachment and Uptake

The initial step for infection by pathogenic mycobacteria is attachment to the macrophage surface and this is accomplished by specific and nonspecific binding to surface receptors. Mycobacteria enter macrophages by both nonopsonic and opsonic modes of phagocytosis. The FcγR and complement receptors are capable of opsonic uptake of mycobacteria. The significance of the FcγR in uptake of pathogenic mycobacteria is not completely clear; however, following antibody coating of mycobacteria there appears to be phagolysosome formation and decreased intracellular survival(9, 106). In contrast, opsonization by complement and uptake via complement receptors may permit these organisms to avoid neutralization mechanisms (79, 136). Nonserum proteins are also capable

of leading to opsonic uptake. Although the precise receptor has not been identified, the pulmonary surfactant, surfactant A, has been shown to increase uptake of *M. tuberculosis.* Uptake of *M. tuberculosis* following coating with surfactant is likely to be due to several factors which includes opsonic phagocytosis (47). An increasing number of nonopsonic receptors have also been described. Nonopsonic binding is likely an important virulence factor for uptake of mycobacteria early in the course of infection or in environments where concentrations of opsonins are low (139). Receptors which lead to nonopsonic uptake of mycobacteria include CR1, CR3, transferrin receptor, and the mannose receptor (100).

Multiple receptors may be used during entry into the cell and the ability to survive intracellularly may to some extent be dependent on the receptors engaged during phagocytosis(9, 72, 78). Overall there is wide variation in receptor use, both opsonic and nonopsonic, among different species of mycobacteria and this is likely related to differences in outer surface components of the bacteria (105). The number of receptors, which have been demonstrated to be used by mycobacteria to gain entrance into macrophages, is large and continues to grow. Two types of receptors important to mycobacterial uptake, the mannose receptor and the complement receptors will be discussed below further.

The mannose receptor

The mannose receptor is an important mode of nonopsonic phagocytosis for several species of mycobacteria including *M. tuberculosis, Mycobacterium bovis (M. bovis)* and *M. avium(100, 105)*. The mannose receptor is transmembrane glycoprotein that acts as a C-type lectin, which is present on the plasma membrane of macrophages; however, it is not present on monocytes (123). There is variability in surface expression of the mannose receptor among these cell types. Nonactivated macrophages tend to have increased expression of

mannose receptor, and in addition to its opsonization properties, surfactant protein A appears to stimulate mannose receptor expression (47). In addition to surface expression of the mannose receptor, there is a large intracellular endosomal pool which undergoes rapid recycling to the cell surface (112).

This mannose receptor binds to ligands with terminal mannose, fucose, and N-acetylglucosamine residues. These carbohydrates tend to be more prevalent on the glycoconjugates of microorganisms than on those of the host thereby facilitating their recognition and uptake by macrophages(71, 94). Virulent strains of *M. tuberculosis* have been demonstrated to use the mannose receptor for uptake into host macrophages(72, 105). One well-characterized *M. tuberculosis* ligand for the mannose receptor is lipoarabinomannan (LAM) (72). LAM is a major surface glycoprotein on the surface of *M. tuberculosis* and in virulent strains the terminal portion is capped with mannose residues (20). Mannose capping of LAM has been demonstrated in other pathogenic species of mycobacteria, while rapid growing nonpathogenic strains typically lack terminal mannose residues (13). Both LAM that is capped with mannose, and LAM that is not, will bind to the lipopolysaccharide (LPS) receptor, CD14, however only capped LAM will bind to the mannose receptor, and uptake via capped LAM may be dependent on interaction with both CD14 and the mannose receptors (13). While mannose receptors will permit uptake of virulent but not avirulent strains of *M. tuberculosis*, it has been demonstrated that the mannose receptor does not discriminate between all pathogenic and nonpathogenic bacteria and will facilitate phagocytosis of both types. This suggests that there are species differences in mannose receptor binding (11).

Selective use of receptors by microorganisms has been suspected as a method of avoiding intracellular killing. Binding and entry via the mannose receptor has been speculated to provide such a "safe haven". Following entry via the mannose receptor by both pathogenic and nonpathogenic species of mycobacteria, failure to activate NADPH oxidase and inhibited phagosome maturation have been demonstrated (11). Although these killing mechanisms were not activated, uptake via the mannose receptor may not completely provide such a "safe haven" as there is evidence suggesting that uptake via the mannose receptor is linked to processing and presentation of LAM to T cells, and that this leads to induction of adaptive T cell responses (96).

Complement receptors

Complement receptors are important for mycobacterial uptake and are capable of initiating both nonopsonic and opsonic mediated phagocytosis. The complement receptors involved in uptake of mycobacteria are CR1, CR3, and CR4 (67) (106). CR 1 is a monomeric transmembrane protein that binds the C3b and C4b fragments. CR3 and CR4 are heterodimers belonging to the β_2-integrin family of adhesion molecules, This family of adhesion molecules is characterized by having identical β-subunits CD18, and variable α-subunits. The α-subunit CR3 is CD11b and CR4 is CD11c. These receptors bind C3bi (39). Following activation of the alternative pathway of the complement cascade C3b and C3bi coated to the bacterial surface will bind to CR1, CR3, and CR4 leading to opsonic uptake(67, 106).

Pathogenic mycobacteria have also developed a strategy to enter macrophages via opsonic uptake mediated by the C2a fragment of C2. This mechanism involves formation of C2a, an enzymatically active C3 convertase, in the absence of C4b, initiating C3b formation

and deposition on the mycobacterial surface from C3 endogenously synthesized in macrophages (107). This mechanism is absent from nonpathogenic species and is a virulence factor specific to pathogenic mycobacteria (107).

M. tuberculosis uptake by human monocytes is not as dependent on serum as other mycobacteria including *M. avium*, and this is due to increased ability of *M. tuberculosis* to promote nonopsonic uptake (122). Nonopsonic uptake of mycobacteria occurs by direct binding of the bacteria with CR3 and CR4(28, 139). A unique binding site, separate from the C3bi binding site on the CR3, has been demonstrated for *M. tuberculosis in vitro,* which binds directly to mycobacteria prior to uptake (113).

The CR3 receptor has a broad range of specificity for many pathogens and, like the mannose receptor, may be associated with increased intracellular survival (38). Ligation of this receptor, in some circumstances, is not associated with activation of the respiratory burst, and has been shown to decrease production of Interleukin-12 by macrophages thus dampening T helper type-I activity and generation of adaptive immunity(79, 136). Recently, an association between CR3 and cholesterol has been described for uptake of mycobacteria by macrophages (46). The cholesterol level within the plasma membrane is responsible for the degree of membrane fluidity (129). *In vitro* evidence suggests that a unique feature of mycobacterial uptake via CR3 is the requirement for cholesterol within the macrophage plasma membrane, and that mycobacteria likely bind directly to cholesterol by interaction with their glycolipid rich cell walls (46).

CR4 expression is increased in macrophages compared to monocytes and has been shown to be an important receptor involved in uptake of *M. tuberculosis*(67, 139). CR4 like CR3 is capable of nonopsonic uptake. Nonopsonic binding of mycobacteria to the CR4

receptor is a powerful activator of signal transduction pathways. Following ligation, there is rapid phosphorylation of a member of the Src family kinases, likely $p60^{src}$ (139). $p60^{src}$ is associated with the cytoskeletal protein paxillin, which is a vinculin-binding protein, and tyrosine phosphorylation of this kinase could result in cytoskeletal rearrangements required for phagocytosis(135, 139).

CR uptake of mycobacteria is also associated with altered Ca^{2+} signaling (78). Increases in cytosolic Ca^{2+} are critical to bactericidal mechanisms including generation of reactive intermediates, cytokine and antimicrobial peptide synthesis(22, 133). Following uptake of *M. tuberculosis* by CRs there is a lack of increase in cytosolic Ca^{2+}, which does not occur when dead *M. tuberculosis* is taken up following ligation with these receptors. Failure to increase cytosolic Ca^{2+} is associated with increased intracellular survival of *M. tuberculosis* and this is another example of the advantage of CR-mediated uptake by mycobacteria (78).

Mycobacterial Inhibition of Phagosomal Acidification

Pathogenic mycobacteria, such as *M. avium*and *M. tuberculosis*, reside in a phagosome with reduced ability to acidify(27, 89). Internalized IgG coated beads or Zymosan are located within a phagosome with a pH of less than 5.5, while that of *M. avium*is between 6.3 and 6.5 (118). The ability to interfere with phagosomal acidification is an important virulence factor as growth of many species mycobacteria including *M. bovis* and *M. tuberculosis* is inhibited when the pH is less than 6.2 (89). An additional benefit of the diminished ability to acidify phagosomal compartments is mycobacterial avoidance of the activated forms of lysosomal hydrolytic enzymes. One such enzyme, cathepsin D, is present within the phagosome of *M. avium*, only in its non-activated higher molecular weight form,

which likely is the result of reduced ability of the phagosome to acidify and cleave cathepsin D into its active form (117).

Mycobacterial exclusion of the V-ATPase proton pump

When compared to phagosomes of *Leishmania mexicana*, which readily acidify, the phagosomes of *M. avium*are lacking the V-ATPase proton pump (118). In addition to phagosomes bearing *M. avium*, those bearing *M. tuberculosis* also lack the V-ATPase within the phagosomal membrane (138). These data suggests that the mechanism responsible for failure to reduce vacuolar pH is exclusion of the V-ATPase from the phagosomal membranes surrounding pathogenic mycobacteria(118, 138). The exact mechanisms for exclusion of V-ATPase are unknown; however, they are likely related to either bacterial inhibition of incorporation of the proton pump into the phagosome, or disassociation of the already formed enzyme within the phagosome. The phagosome containing *M. avium*lacks both the 56 and 31kDa subunits of the V-ATPase, which suggests that mycobacteria inhibit incorporation or the retention of the proton pump, rather than dissociation after the pump has been assembled within the phagosomal membrane (118). The ability to inhibit phagosomal acidification by *M. avium*and different strains of *M. tuberculosis* was similar, even though there is marked variation in virulence among these organisms (138).

The phagosomes which contain pathogenic mycobacteria are markedly less acidic than those containing corresponding dead bacteria; however, these phagosome still have a pH which is lower than the cytosol (118). This decreased pH suggests some type of acidification is occurring within the mycobacterial phagosome. Other proton pumps including the NHE, and the Na^+/K^+-ATPase are possibilities, however the acidification is completely inhibited by Bafilomycin A_1 indicating that some V-ATPase activity is responsible for decreases in

phagosomal pH(60, 118). The source of this residual V-ATPase activity is unknown. One possibility is that during phagosomal formation some V-ATPase within the plasma membrane is incorporated into the budding phagosome which is subsequently removed (31). A continuous H^+ leak occurs in these phagosomes so the pH needs to be maintained by continuous pumping, which does not support the idea that the V-ATPase is transiently present and originates from the plasma membrane (60). A second possibility is that pH may be maintained by fusion with other vesicles with reduced pH, but that lack V-ATPase within their membranes (60). The decrease in mycobacterial phagosomal pH appears to dissipate with increased time and as mycobacteria replicate their phagosomes appear to divide and become less acidified(31, 138).

Although low pH is detrimental to mycobacterial survival there is variation among the different species in their ability to tolerate acidic environments. Coinfection of murine macrophages with either *M. tuberculosis* or *M. avium.* and *Coxiella burnetii* leads to acidification of the corresponding mycobacterial phagosomes. Using this technique *M. tuberculosis* was found to be more susceptible to acidic environments compared to *M. avium*(48). This enhanced sensitivity of *M. tuberculosis* to low pH compared to *M. avium* has also been demonstrated by severe growth restriction of *M. tuberculosis* in mildly acidic media, while *M. avium* grew much more readily (91).

Role of Nramp-1 in resistance to mycobacterial infections

Natural resistance-associated macrophage protein-1 (Nramp-1) influences phagosomal acidification during infection by certain species of mycobacteria. Nramp-1 is a 110 kD integral membrane protein expressed nearly entirely within macrophages, which is encoded by the mouse *Bcg/Ity/Lsh* locus (56). Nramp1 is associated with innate resistance to

several intracellular pathogens including *M. bovis*, *Salmonella typhimurium*, and *Leishmania donovani* (110). Based on structural similarities between the Nramp family of proteins and transporter and channel proteins, it is likely that Nramp1 acts as a transporter of small charged ions (132).

By using knockout mice deficient in Nramp-1 it was shown that *M. bovis* (BCG) phagosomes are significantly less acidic in Nramp-1 knockout mice than in the corresponding phagosomes of wild type mice. This indicates that the role of Nramp-1 in resistance to intracellular pathogens is associated with alteration of phagosomal pH (61). The mechanisms by which Nramp-1 increases phagosomal acidity are incompletely understood, however it appears that increased phagosomal acidity is due to increased V-ATPase activity (61). The Nramp-1 activity is specific only for phagosomes of live BCG since those of killed bacteria had a similar level of acidification in both wild type and Nramp-1 deficient mice (61).

While a protective role for Nramp-1 has been identified against BCG and several other species of mycobacteria, including *M. avium* in mice, it does not appear to play a significant role in defense against *M. tuberculosis*. (7, 87). When wild type and Nramp-1 deficient mice are infected with virulent strains *of M. tuberculosis*, the wild type mice demonstrate equal if not increased susceptibility to infection over those mice deficient in Nramp-1(82, 87). These studies further illustrate the variability in the interactions and susceptibilities of different species of pathogenic mycobacteria within macrophages. An example of this is illustrated by *M. avium* which is less susceptible than *M. tuberculosis* to the toxic effects of phagosomal acidity; however, it is more susceptible to the effects of Nramp-1 (48).

Cytokine effects on mycobacterial phagosomal acidification

Macrophage activation affects ability of the mycobacterial phagosome to resist acidification. Macrophages that are activated by cytokines including TNF-α , IFN-λ, and IL-1 have increased capability in controlling mycobacterial infection both *in vitro* and in *vivo*(26, 42, 104). Although several factors of phagosome maturation are affected following cytokine activation, one mechanism which increases the macrophage's ability to control intracellular infection is the increased ability to acidify the mycobacterial phagosome. Following activation of murine bone marrow derived macrophages by IFN-λ and LPS the pH of the phagosomes containing *M. avium* dropped to 5.2, and this lower pH was associated with increased V-ATPase labeling on the mycobacterial phagosomes (104). Increases in phagosomal acidity following macrophage activation by IFN-λ and LPS can be inhibited by the antiinflammatory cytokine IL-10, which suggests that mycobacterial survival is affected by pro and antiinflammatory cytokines in the local environment (131).

Mycobacterial Phagosome Maturation

Early studies of intracellular trafficking of pathogenic mycobacteria demonstrated that their phagosomes remain separate from lysosomal compartments, and the ability to do this is a major survival strategy for mycobacteria (figure 1)(10, 32). Included in these studies were data indicating that virulence of the strain of mycobacteria correlated with ability to avoid phagosome-lysosome fusion. These studies demonstrated that the phagosomes of virulent *M. tuberculosis* did not fuse with ferritin-labeled lysosomes; however, fusion occurred between labeled lysosomes with the phagosomes of damaged organisms or those with decreased virulence (63). This was further supported by studies with selectively labeled lysosomes (with staining for acid phosphatase) and prelysosomal compartments (horse radish

peroxidase) in *M. avium* infected cultured macrophages, and here inhibition of fusion between both lysosomal and prelysosomal vesicles was observed (43). With the description of different models for trafficking of endosomes and phagosomes to their corresponding lysosomal stages, the term "phagosome-lysosome fusion" may not be completely accurate. According to the "maturation model" this may be better termed "phagolysosome maturation", and according to the "kiss and run" and vesicular shuttle" models lysosomes have only transient interaction with earlier vesicular compartments(85, 114, 124).

Interaction between mycobacterial phagosomes and early endosomes

In contrast to early studies where the phagosomes of pathogenic mycobacteria were thought to be static and isolated, these phagosomes are in a dynamic state and continue to interact with early endocytic compartments. Following uptake it has been demonstrated that the mycobacterial phagosome integrates into the population of early endosomes, intermingling contents and membranes(31). As the transferrin receptor does not traffic to late endosomes or lysosomes, exogenously administered transferrin is specific for early endosomes(24, 74).

When exogenously administered, transferrin accumulates within the phagosomal membranes of live, but not dead, *M. tuberculosis,* and within the *M. avium* phagosome. The amount of transferrin within these mycobacterial phagosomes was variable and often low, but is enriched over those of dead mycobacteria or IgG coated bead phagosomes(24, 117). There are three potential sources of the transferrin receptor: the plasma membrane, the trans-Golgi network, or early endosomes. Although some of the transferrin receptor may have been taken in from the plasma membrane, these studies indicate that the transferrin receptor is delivered to the mycobacterial phagosome by interaction with early endosomes since

exogenously administered transferrin would not be expected to accumulate here if its source was the trans-Golgi (24). These studies indicate that the phagosomes of *M. avium* and *M. tuberculosis* represent arrest in a normal stage of phagosomal processing with continued interaction of the mycobacterial phagosome and early stages of the endosomal network, and like non-matured endosomes, these non-matured phagosomes are unable to fuse with lysosomes(32, 117).

A lack of communication of mycobacterial phagosomes with the late endocytic compartments is demonstrated by studies where *M. avium* and *M. tuberculosis* phagosomes in infected macrophages did not acquire endocytic tracers (Man-BSA, BSA-gold) which target lysosomal compartments(23, 138). During the course of intracellular infection the phagosomes of *M. avium* and *M. tuberculosis* release large amounts of LAM via budding off from their phagosomal membranes. Despite loss of membrane from these buds the mycobacterial phagosomal membrane remains intact suggesting that the lost membrane must be replaced by fusion with other vesicles (138). The phagosomal membranes of both *M. avium* and *M. tuberculosis* are also readily accessible to the glycosphingolipids, which are hydrophobic oligosaccharide moieties that form part of the glycocalyx of the plasma membrane. These lipids are internalized and may be processed in lysosomes; however, a significant amount are recycled back to the cell surface (102).

The mycobacterial phagosome is not static but has evolved a method for remaining in a continuous state of selective fusion with endosomal compartments that maintain the phagosomal membrane, and leads to a trafficking pattern mimicking recycling endosomes (117). Endosomes involved in recycling have limited ability to acidify, which is a similar to the environment in the mycobacterial phagosome (102). In a conflicting study where

mycobacterial phagosomes were directly isolated from murine macrophages and subjected to 2-D electrophoresis, the composition of the phagosomes very closely resembled the plasma membrane. This led to the suggestion that mycobacterial phagosomes, while dynamic, acquires protein markers randomly from the cell surface during internalization and remain separate from the endosomal pathway (65).

Mycobacterial phagosomal membrane markers

The phagosomes containing pathogenic mycobacteria retain markers specific for early endosomes, while those of late endosomes and lysosomes tend to be excluded. The phagosomes of nonpathogens initially contain markers of the early endosomal compartment but eventually lose these markers and acquire those of lysosomes (75). The phagosomal membrane of *M. tuberculosis* contains increased amounts of the early endosomal markers MHC I and MHC II, and the transferrin receptor, while relatively low amounts of late endosomal markers CD63, Lamp-1 and Lamp-2 (23). The mannose 6-phosphate receptor which is a marker for lysosomal trafficking is absent from the phagosomes of *M. tuberculosis* and *M. avium* (138). In addition to these markers, the phagosomal membranes of pathogenic mycobacteria are enriched in the early endosomal marker Rab5 and are deficient in Rab7, a marker specific for late endosomes (130).

Mycobacterial phagosomes are not completely devoid of late endosome-lysosomal markers, especially Lamp-1, and reports differ as to the significance and mechanisms of delivery of Lamp-1 to mycobacterial phagosomes. Delivery of this marker appears to be an early event following formation of the mycobacterial phagosome, occurring prior to acquisition of the V-ATPase pump, and may represent an alteration in trafficking of the endosomal pathway prior to the lysosomal stage(34, 118). Lamp-1 has been identified by

some to be within the mycobacteria phagosomal membrane in low to moderate amounts, however in much lower concentration than that identified within the phagolysosomes of beads or dead bacteria(12, 23, 25). In contrasting reports, the phagosome of *M. tuberculosis* has been found by some to contain considerable amounts of Lamp-1 (138). Demonstration of Lamp-1 protein within the mycobacterial phagosome does not necessarily mean that it is present in as high a concentration as is found in lysosomes, where Lamp-1 completely lines the inner surface of the lysosomal membrane(34, 45). It may be that not only the presence of Lamp-1, but also concentration within the phagosome is required in order to mark a late phagosome/endosome stages.

Delivery of this marker to the mycobacterial phagosome may occur by different mechanisms. Lamp-1 is present of the plasma membrane of certain cell types, which may be taken up during formation of the mycobacterial phagosome(45, 65). During normal trafficking Lamp-1 reaches the lysosome by two main pathways. The first is transport from the trans-Golgi, following synthesis, initially to endosomes then to lysosomes. In the second method of transport, Lamp-1 is delivered to the cell surface from the trans-Golgi followed by internalization and transport to the lysosomes by the endocytic pathway (101). Mycobacterial phagosomes then may acquire Lamp-1 by interaction with these Lamp-1 containing endosomes (34). Additionally there are likely differences in Lamp-1 acquisition by mycobacterial phagosomes in different macrophages cell lines (34, 65)

Mycobacterial inhibition of phagolysosome formation

The mechanisms by which pathogenic mycobacteria are able to avoid phagolysosome formation are incompletely understood; however, multiple factors likely play a role. The ability to resist phagolysosome formation appears to be an energy requiring process that is

dependent on viability of the bacteria (dead mycobacteria are transported normally along the phagosomal pathway with subsequent phagolysosome formation)(10, 12, 65). Chemical mediators of mycobacterial origin, such as ammonia, ammonium chloride, and sulfatides, have been incriminated as inhibitors of phagosome-lysosome interaction(49, 50, 64). Alteration in gene expression with increased expression of certain proteins has also been demonstrated coinciding with intracellular growth of *M. avium*, which may be related to inhibition of phagosomal maturation (116).

A novel host protein termed tryptophane aspartate-containing molecule or TACO has been shown *in vitro* to be retained within the phagosomes of pathogenic mycobacteria (41). In normal phagosomal trafficking this protein is lost prior to phagosome fusion with lysosomes. Live but not dead pathogenic mycobacteria however are capable of retaining TACO within their phagosomal membranes thereby preventing phagolysosome formation, which is likely by interference with the fusion machinery recruited to the phagosomal membrane prior to fusion with lysosomes (41). In addition to facilitating uptake of mycobacteria, cholesterol plays a role TACO recruitment. Uptake via cholesterol domains appears to be a prerequisite for mycobacterial sequestration within TACO bearing phagosomes (46).

Increases in cytosolic Ca^{2+} following phagocytosis are required for the signal transduction pathways that lead to phagosome-lysosome interaction, and this elevation of Ca^{2+} has been demonstrated to be depressed following mycobacterial uptake by complement receptors in human macrophage derived monocytes. This failure to raise cytosolic Ca^{2+} is likely to be a factor in failure of phagolysosome development (78). It has also been demonstrated in murine macrophages that following infection with *M. avium* there is

pronounced disruption of the actin filament network approximately 1 day into the infection, however this does not appear to have a direct effect on mycobacterial phagosomal maturation (58).

The ability to inhibit interaction with lysosomal compartments has also been shown to be related to factors independent of mycobacteria. Included in these are the cell line or species infected, and the degree of activation of the host macrophage (30, 131). Attention has recently turned to two areas which appear to have a direct influence on phagosome maturation: fusion facilitators which confer specificity to docking and fusion events between phagosomes and endosomes present on the mycobacterial phagosome, and the physical properties of pathogenic mycobacteria(33, 34).

Rab proteins and the mycobacterial phagosome

Disruption of the acquisition of Rab proteins appears to play a key role in inhibition of phagolysosome development. Rab proteins are fusion facilitators, which with additional membrane proteins such as SNAPs and SNAREpins, regulate endosome/phagosome maturation. 30 different Rab proteins have been identified and each likely has a role in fusion in different compartments of the endocytic and secretory pathways (25). Rab 5 is found within the plasma membrane, within membranes of early endosomes, and is present within the phagosome immediately following phagocytosis and facilitates fusion between phagosomes/endosomes with early endosomes(21, 51). Rab7 is associated with late endosomes and facilitates fusion events between late endosomes (36). Typically particles that are phagocytosed, such as latex beads, will reside within a phagosome which initially acquires Rab5, followed by loss of Rab5 and acquisition of Rab7 within the phagosomal membrane (36).

The phagosomes of pathogenic mycobacteria retain Rab5 and fail to incorporate Rab7 within their membranes (25, 130). By retaining Rab5, the mycobacterial phagosome is capable of continued interaction with early endosomes and by exclusion of Rab7 is unable to fuse with later endosomes thus inhibiting the maturation of these phagosomes to phagolysosomes (25). This dysregulation of Rab proteins suggests that pathogenic mycobacteria do not actively inhibit phagosome-lysosome fusion, but rather, through the retention and exclusion of specific fusion facilitators, allow for continued phagosome-endosome fusion thus preventing phagosome-lysosome fusion (117).

Physical properties of mycobacteria and phagolysosome development

The physical properties of pathogenic mycobacteria participate in interference of phagosomal maturation. Phagosomes that contain degradable particles interact as expected with early and late components of the endocytic compartments eventually obtaining markers of phagolysosomes, while those that resist degradation tend to remain fusogenic only to early endosomes (31). Pathogenic species of mycobacteria reside within a phagosome and resist degradation and, like latex beads, continue to interact with early endosomal compartments thus resisting maturation into a phagolysosome (31).

The closeness of the apposition of the phagosomal membrane with the phagocytosed particle also affects phagosomal maturation. A tight fit between the phagosome and particle occurs if the particle has a hydrophobic surface, and in contrast a loose fit occurs if the particle has a hydrophilic surface. The tight fitting phagosomal membrane tends to retard phagosomal maturation (33). The fit of the phagosomal membrane around nonopsinized mycobacteria is tight and resists maturation, whereas there is a loose fit of the phagosome around antibody coated mycobacteria and this opsonization leads to phagolysosome

formation (33). The closeness of the apposition of the phagosome and the particle likely interferes with phagosomal maturation events and restricts membrane intermingling between vesicles (33).

Cytokines and mycobacterial phagosomal maturation

Following activation of macrophages there is decreased ability of the mycobacterial phagosome to resist maturation and phagolysosome formation; however, there is variable correlation with cytokine activation of macrophages and increased intracellular bacterial killing. Following activation by LPS and IFN-γ, retention of markers associated with early endosomes are lost on the mycobacterial phagosome. The transferrin receptor, which is associated with the phagosomes of pathogenic mycobacteria, is no longer present in appreciable amounts within mycobacterial phagosomes following activation. In addition, following activation there are increased concentrations of the lysosomal marker Lamp-1 within the mycobacterial phagosome. These changes in phagosomal markers indicate that the mycobacterial phagosome is shifted toward later stages of the phagosome maturation (104). This shift precedes actual decreases in viability of the bacteria, and ultrastructurally one of the first changes noted is fewer mycobacteria within individual vacuoles and more vacuoles that have merged to contain several viable bacteria (104).

Loss of viability of mycobacteria is likely a gradual process following macrophage activation and phagolysosome formation, and death is due to the transition from a relatively safe environment to the hostile environment of the lysosome characterized by lower pH, acid hydrolases, and oxygen and nitrogen reactive intermediates (104). Even in these conditions pathogenic mycobacteria such as *M. tuberculosis* may not be killed but remain static with potential for reactivation (104).

Although activation of macrophages may lead to increased killing of mycobacteria, macrophages infected with mycobacteria tend to be more refractory to activation by IFN-λ than noninfected macrophages (69). *M. avium* infection of murine macrophages leads to down-regulation of the IFN-λ receptor, which leads to failure to activate IFN-λ inducible genes and subsequent signal transduction pathways including Janus kinase-Stat signaling (69). *In vitro* studies in primary bovine monocytes demonstrated variable ability of IFN to restrict intracellular growth of *M a. ptb*, however using crude cytokine extracts derived from *M. a. ptb*-immune mononuclear cell culture supernatants (IMCS) intracellular growth of the bacteria was actually enhanced (140). These data suggest that bovine macrophages are fairly refractory to the effects of cytokine-mediated activation and restriction of intracellular growth of *M. a. ptb*, unless adequate amounts of cytokines (such as IFN) are present (140). Activation of macrophages by cytokines derived from lymphocytes, NK cells, and other macrophages is not the only method by which macrophages can become more efficient at killing intracellular mycobacteria. Endothelial cells of the microvasculature play an important role in inflammation and infection, and represent the interface between immune cells and sites of infection(90) .When macrophages are incubated with microvascular endothelial cells there is increased intracellular killing of *M. avium*, and this has been shown to be due to stimulation of the macrophage by platelet activating factor released from the endothelial cells (90). Platelet activating factor has also been shown to reverse mycobacterial suppression of cytosolic Ca2+ following phagocytosis, which indicates that infection with mycobacteria does not disable Ca2+ mediated signal transduction (78).

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CHAPTER TWO. CHARACTERIZATION OF ACIDIFICATION OF PHAGOSOMES CONTAINING *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN J774 CELLS

A paper prepared for submission to *Veterinary Pathology*

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Abstract

Mycobacterium avium subspecies *paratuberculosis* (*M. a. ptb*) is the causative agent of Johne's disease, a progressive diarrheal disease of wild and domestic ruminants. One mechanism developed by several pathogenic species of mycobacteria for intracellular survival is inhibition of acidification of the phagosomal compartment in which they reside. We hypothesize that following phagocytosis *M. a. ptb* resides within a phagosomal compartment that has reduced ability to acidify. To test this hypothesis, macrophages containing live *M. a. ptb* were compared to macrophages containing killed *M. a. ptb*, Zymosan A, or the non-pathogenic species *Mycobacterium smegmatis* (*M. smegmatis*) in J774 monolayers at 2, 6, and 24 hours postinfection periods. Colocalization of fluorescent signals generated by direct conjugation of bacteria with fluorescein isothiocyanate (FITC) and labeling acidified compartments within macrophages with Lysotracker Red, was identified by laser confocal microscopy. Colocalization was also evaluated after addition of the specific V-ATPase inhibitor Bafilomycin A_1. In addition, acidification of the phagosome containing *M. a. ptb* was evaluated by transmission electron microscopy following labeling of acidified compartments with the weakly basic amine (3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine (DAMP) with subsequent immunogold labeling. The results of this

study demonstrate that, compared to Zymosan A, killed *M. a. ptb*, or *M. smegmatis*, there was reduced colocalization of live *M. a. ptb* with Lysotracker Red by confocal microscopy and reduced accumulation of DAMP within the phagosomal compartment containing live *M. a. ptb*. These results indicate that like other species of pathogenic mycobacteria, *M. a. ptb* resides within a phagosome that resists acidification. There was nearly complete loss of colocalization of Lysotracker Red with killed *M. a. ptb*, zymosan A, and *M. smegmatis* following treatment with Bafilomycin A_1. This demonstrates dependence of Lysotracker Red labeling on V-ATPase and suggests that the mechanism by which *M. a. ptb* inhibits phagosomal acidification is exclusion of the V-ATPase proton pump from the phagosomal membrane.

Key words: *Mycobacterium avium* subspecies *paratuberculosis*, phagosomal acidification, Lysotracker Red, DAMP, J774 cells

Introduction

Mycobacterium avium subspecies *paratuberculosis (M. a. ptb)* is the causative agent of Johne's disease, a progressive diarrheal disease of domestic and wild ruminants (see appendix one for review). *M. a. ptb* is an intracellular pathogen of mononuclear phagocytes, which has been shown to survive and replicate within bovine and ovine monocytes and monocyte-derived macrophages(24, 26). Intracellular pathogens have developed several strategies to prevent their destruction by phagocytes that include escape into the cytoplasm, inhibition of phagosomal acidification, and inhibition of phagolysosome formation.

The mechanisms by which *M. a. ptb* is capable of survival and replication within macrophages are not well-characterized. Following uptake into macrophages, particles are typically contained within a phagosomal compartment that quickly develops into a potent antimicrobial environment. In contrast, both *Mycobacterium tuberculosis* and *M. avium* reside within a phagosomal compartment that resists the normal sequence of phagosomal development which follow phagocytosis, thereby avoiding the destructive mechanisms associated with phagosome maturation(2, 6, 16). One of the mechanisms used by *M. tuberculosis* and *M. avium* is inhibition of phagosomal acidification(7). *M. a. ptb* has a genome which has been shown to have approximately 90% homology with *M. avium*, and as a result has been classified as a subspecies of *M. avium*(5, 12, 13). Considering the close phylogenetic relationship between *M. avium* and *M. a. ptb*, the mechanisms used for intracellular survival by these two pathogens may be similar.

In the current study we characterized acidification of the phagosomal compartments of *M. a. ptb*. The hypothesis of this study, that the phagosomes containing *M. a. ptb* have diminished ability to acidify, was tested by comparing the phagosomal pH of live *M. a. ptb* with those of killed *M. a. ptb*, yeast cell wall extract (zymosan A), and the nonpathogenic mycobacterium *M. smegmatis* taken up by macrophages. For this study, the murine macrophage cell line J774 was used. The ability of *M. a. ptb* to survive and replicate within this cell line has been previously described (20).

Compartments with low pH were labeled by the addition either Lysotracker Red or (3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine (DAMP). Both of these compounds selectively accumulate within intracellular compartments with low pH, where they are chemically modified and retained, and both have been demonstrated to be reliable markers of

phagosomal acidification (1, 15, 23). Colocalization signals generated by the Lysotracker Red and fluorescein isothiocyanate (FITC) labeled bacteria were identified by laser confocal microscopy. Transmission electron microscopy was then used to identify immunogold labeled DAMP within the phagosomes of live and dead *M. a. ptb* in order to further support the confocal findings. To determine the role of V-ATPase in phagosomal acidification by live and dead *M. a. ptb*, Zymosan A, and *M. smegmatis*, colocalization of these agents with Lysotracker Red was assessed following the addition of a proton pump inhibitor, Bafilomycin A_1 (14).

Materials and Methods

Materials: Lab-Tek II chambered glass slides were purchased from Nalge Nunc International (Naperville, IL). Millipore culture plate inserts (.45 μM, 12mm in diameter) were purchased from Millipore Products Division (Bedford, MA). Lysotracker Red ™, and LIVE/DEAD® Bac*Light*™ kits were purchased from Molecular Probes(Eugene Oregon). The V-ATPase proton pump inhibitor Bafilomycin A_1 and yeast wall extract Zymosan A were both purchased from Sigma Chemical Company (St. Louis, MO). DAMP [3-(2,4-dinitroanilin)-3'-amino-N-methyldipropylamine] and monoclonal anti-DNP (clone HDP1) were from Oxford Biomedical Research, Inc (Oxford, MI). Gold conjugated goat anti-mouse was purchased from Ted Pella Inc. (Redding CA).

Bacterial Strains and Growth Conditions: The standard American Type Culture Collection (ATCC) strain of *Mycobacterium avium* subspecies *paratuberculosis (M. a. ptb)* 19698, was obtained from the USDA/ARS National Animal Disease Center, Ames, Ia. *Mycobacterium smegmatis* was obtained from ATCC (ATCC 607). Bacteria were grown in

Middlebrook's 7H9 broth supplemented with Middlebrook's OADC enrichment and Mycobactin J. Bacteria were passed into fresh broth weekly to maintain log phase growth. Inoculum was prepared by sedimenting bacteria via centrifugation at 3000 x *g* for 10 min, and then resuspending the pellet in sterile .15M NaCl. Repeated passage of the bacterial suspension through a 25-gauge needle (at least 10X) immediately prior to inoculation of monolayers greatly reduced the tendency for bacteria to clump. The result was an approximately 85-90% single cell bacterial preparation. Bacterial numbers were determined by measuring absorbance of the bacterial suspension at 540 nM and comparing values to graphical data of colony forming units of *M. a. ptb* vs absorbance at 540nM. Bacterial viability was determined by using the Molecular Probes LIVE/DEAD® Bac*Light*™ kit as instructed by the manufacturer. After labeling, live bacteria fluoresce green and dead bacteria red. One hundred bacteria in at least 10 different high power fields (40X) were visually counted by fluorescent microscopy (Olympus, Melville, NY) and the results expressed as a percentage.

Macrophage Cell Line: The mouse macrophage cell line J774A.1 (ATCC TIB-67) was maintained in a humidified, 37 °C, 5% CO_2 incubator. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal calf serum, 4 mM L-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate. Macrophages were seeded onto chambered slides at 1.0 x 10^5 cells per ml and placed in the incubator overnight prior to confocal microscopy studies. For evaluation by electron microscopy, 1.0 x 10^5 cells per ml were seeded into Millipore culture plate inserts (0.45 μM 12mm in diameter) overnight prior to infection.

Infection of Macrophages: Prior to confocal studies mycobacteria and zymosan A were conjugated with fluorescein isothiocyanate (FITC) by a modification of a method previously described (3). Briefly, 4 x 10^7 broth grown bacteria or zymosan were suspended in .001% FITC in a .2 M Na_2CO_3-$NaHCO_3$ buffer containing 150 mM NaCl (pH 9.2) for 30 minutes. Bacteria were then washed twice in saline and finally resuspended in Dulbecco's Modified Eagle's Medium. Bacteria were added to the monolayers of adherent macrophages in chambered slides with a final multiplicity of infection (MOI) of 10:1. Bacteria were incubated with monolayers for 2, 6, and 24 hours. For transmission electron microscopy, the incubation period was 2 hours. Bacteria were not labeled prior to infection, and monolayers were infected with either live or dead *M. a. ptb* (same MOI as for the confocal studies).

Lysotracker Red: DMEM containing 50 nM Lysotracker Red was added to the chambered slides during the last 60 minutes of incubation. Following incubation, monolayers were washed with two exchanges of medium (wash medium contains 50 nM Lysotracker Red) to remove any extracellular bacteria. Any remaining extracellular FITC fluorescence was quenched by flooding the slide with 1 % tannic acid for one minute, followed by two washes with medium (10). Following the last wash, slides were coverslipped and were evaluated immediately following preparation to minimize the fading associated with Lysotracker Red.

DAMP Assay: Labeling by the DAMP technique was done as previously described (22): After the incubation period, cells were washed once with phosphate buffered saline (PBS) (37^{0}C). Inserts were then filled with 0.8ml of DMEM containing 60 μM of DAMP and allowed to incubate for 5 minutes. Medium was then aspirated and cells washed once with PBS (4^{0}C). Membranes were then fixed in 1% glutaraldehyde in Dulbecco's PBS (pH 7.2)

for 15 minutes. Medium was then aspirated and the inserts washed three times with Dulbecco's PBS. Cells were then incubated with .5M NH_4Cl for 30 minutes to quench residual aldehydes. Cells were then washed three times in Dulbecco's PBS. Post-fixation was for 1 hour at room temperature in a 1% osmium tetroxide in 100mM Na_2HPO_4 (pH 7.2) followed by 3 washes in phosphate buffer. Cells were incubated for 30 minutes at room temperature in 0.01% tannic acid then washed three times in phosphate buffer. Cells were infiltrated and embedded in epoxy resin. Membranes were sectioned at 80nm and placed on nickel grids (nickel square 200 mesh grid, 3.05 mm- Polysciences, Inc. Warrington PA). Immunolabeling procedure was as follows: Sections were etched for 30 minutes in a saturated solution of sodium metaperiodate n water, washed in water, dried and incubated for 1 hour in 1% ovalbumin (OA)(Sigma) in tris buffer (pH 8). Grids were then placed in 1% OA containing 5ug/ml of anti-DNP antibody (which binds to DAMP), and incubated for 18 hours at 4°C. The grids were then washed in tris buffer containing 1% OA and were then placed in a 1:10 solution of gold labeled anti mouse secondary antibody (Ted Pella, Inc. Redding, CA) in a .1% OA in tris buffer for 2 hours at 37^{0}C. Grids were then washed with tris buffer containing .1% OA then washed in boiled distilled water and dried. Grids were stained with 2% uranyl acetate and 2.5% Reynolds lead citrate. Control inserts were prepared (cells infected with live and killed *M. a. ptb*) by omitting the addition of DAMP.

Bafilomycin A_1: J774 monolayers were seeded onto chambered slides as previously described for confocal microscopy(23). Monolayers were then incubated with DMEM containing .25nM Bafilomycin A_1 for one hour, then infected with either live or dead *M. a. ptb*, Zymosan A, or *M. smegmatis* for 2, 6, and 24 hours. Bafilomycin A_1 was maintained in the media throughout the incubation periods.

Laser Confocal Microscopy and Colocalization Studies: The peak emissions for FITC and Lysotracker Red are 518 nm and 592 nm respectively, which allowed for spectral separation of these two fluorescent signals by the confocal microscope. Colocalization signals were generated as follows: When FITC signal from labeled bacteria were not superimposed (noncolocalized) with Lysotracker Red labeled acidified compartments they retained their green fluorescence; however, when bacteria and low pH compartments were superimposed (colocalization), the overlap generates a yellow signal. Bacteria were then visually scored as being colocalized (yellow) or non-colocalized (green). Determination of colocalization of bacteria and lysosomes was accomplished by a modification of a system described for sampling intracellular bacteria by confocal microscopy (4). Images were captured by analyzing slides with a Leica TCS-NT confocal microscope (Leica Microsystems, Inc., Exton PA) equipped with a krypton (568 nm-excitation wavelength) argon (488 nm-excitation wavelength) and HeNe (633 nm-excitation wavelength) lasers. Colocalization of FITC labeled bacteria or zymosan with Lysotracker Red labeled acidic compartments was accomplished by the following method. For every time point, at least 50 individual bacteria/zymosan were counted in at least 10 random fields for each slide. Images were then merged by IPlab (Scanalytics, Fairfax, VA.). Signals were scored only if they were clearly discernible as green or yellow and had morphology consistent with the corresponding particle. The Few signals which were difficult to identify as green or yellow were discounted. Results were expressed as a percentage and were based on at least two replicates per experiment. Surface bacteria were excluded by quenching their fluorescence with tannic acid.

Electron Microscopy: Samples were examined at 34,000x with a Philips 410 transmission electron microscope (FEI Company, Hillsboro, OR). For live or dead *M. a. ptb*

and control samples, bacteria were photographed in at least 8 phagosomal compartments of similar size in 5-8 cells per group. The numbers of gold particles were counted for each phagosomal compartment, and the results expressed as average number of gold particles per phagosome. This study was done once and results are based on this one replicate.

Statistical Analysis: Data were expressed as percent colocalization of bacteria or yeast with Lysotracker Red for confocal studies. Sources of variation were tested by General Linear Models procedure using SAS statistical software (SAS Institute, Cary, NC). Results were deemed significant at $P < 0.05$.

Results

Bacterial Viability: Prior to labeling and infection of monolayers, viability of live *M. a. ptb* and *M. smegmatis* were determined by the Bac*Light*™ kit . Approximately 90% and 70% of the *M. a. ptb* and *M. smegmatis* inocula, respectively, were found to be viable. Approximately 80% of the heat killed *M. a .ptb* gave a red fluorescent signal by the Bac*Light* Live/Dead kit which indicates dead bacteria. FITC conjugation of live *M. a. ptb* and *M. smegmatis* did not affect bacterial viability, which was demonstrated by colony formation on 7H10 slants in similar numbers as non-conjugated bacteria (data not shown).

Confocal Evaluation of Phagosomal Acidification: This study demonstrated that the extent of colocalization of live *M. a. ptb* with Lysotracker Red was significantly decreased compared to those of killed *M. a. ptb*, *M. smegmatis*, or Zymosan A (Table 1, Figure 1). The mean percentage of colocalization of live *M. a. ptb* was 52.2 % for all time points, whereas the mean percentage of colocalization of dead *M. a. ptb*, *M. smegmatis*, or zymosan A with low pH compartments was 79.3 %, 69.7, and 88.2 % respectively

(summarized Table 1). Colocalization was evaluated at 3 time points; however no significant differences in percent colocalization were noted for live *M. a. ptb* or the other agents. Zymosan A served as the positive control and as expected had a high degree of colocalization with Lysotracker Red at all time points (mean 88.2 %). Heat killed *M. a. ptb* demonstrated similar colocalization with Lysotracker Red as Zymosan A. The differences in percentage colocalization of *M. smegmatis* with killed bacteria were not significant; however the difference between *M. smegmatis* and zymosan A were significant, and *M. smegmatis* had an intermediate frequency of colocalization between that of live and killed *M. a. ptb* (Table 1).

Inhibition of V-ATPase: Treatment of the J774 cells with Bafilomycin A_1 prior to infection lead to a uniform drop in the percent colocalization of live and heat killed *M. a. ptb*, *M. smegmatis*, and zymosan A with Lysotracker Red to between 0-5% (Figure 2).

Electron Microscopy: There was reduced immunogold labeling of DAMP within phagosomes containing killed *M. a. ptb* compared to phagosomes containing live *M. a. ptb* (Figures 3). There were an average of 49 gold particles per phagosome for killed *M. a. ptb* compared to an average of 23 gold particles per phagosome for live *M. a. ptb*. Phagosomes within the control sample (killed bacteria with DAMP addition omitted) had very little immunogold labeling with 2 gold particles per phagosome.

Discussion

The results of this study demonstrate that the phagosomal compartments containing *M. a. ptb* have diminished ability to become acidified. This ability to manipulate phagosomal acidification serves as an important virulence factor for *M. a. ptb* because acidification contributes to decreased intracellular survival by being directly toxic, by activation of

hydrolytic and proteolytic enzymes, and is required for further phagosomal maturation(11, 18, 21). Phylogenetically, the relationship between *M. a. ptb* and *M. avium* has been shown to be very close, and the ability of *M. a. ptb* to inhibit acidification of its phagosomal compartment is consistent with that observed for *M. avium* (12, 13). In studies comparing *M. avium* to both *Leishmania* spp. or zymosan A, it has been shown that the pH of phagosomes containing *Leishmania* or zymosan A rapidly dropped to 5.5 or lower, while those of *M. avium* remained at 6.3 to 6.5 (21). In addition to *M. avium,* our data is in agreement with reports showing that other pathogenic species of mycobacteria, including *M. tuberculosis*, *M. bovis*, and *M. marinum,* reside within macrophages in nonacidified phagosomes(7, 11).

We demonstrated reduced acidification of the *M. a. ptb* phagosome by using a two color fluorescent imaging system. Direct fluorescein isothiocyanate (FITC) conjugation of bacteria was a rapid and reliable method for labeling which, consistent with other reports using this technique, did not affect bacterial viability(3, 7, 11, 16). Lysotracker Red is a highly selective marker of acidic organelles with the advantage of being able to identify compartments of low pH with minimal luminal content. This was an important feature considering that the phagosomal membrane has been shown to be very closely opposed to the surface of pathogenic mycobacteria leaving little remaining luminal space(9, 23).

Using confocal microscopy we detected a marked decrease in intracellular colocalization signals generated by overlap of Lysotracker Red with FITC labeled live *M. a. ptb* compared to those of killed *M. a. ptb*, *M. smegmatis*, and zymosan A (Figure 1). Reduced colocalization indicates that increased numbers of live *M. a. ptb* were within phagosomal compartments that did not retain Lysotracker Red, suggesting impaired acidification of these phagosomes. While colocalization of Lysotracker Red with live *M. a. ptb* was not abolished,

it was significantly reduced (P>.05) (Table 1). The results of our study did not show an effect of time on acidification of the *M. a. ptb* phagosome; however, others have identified a transient acidification of the mycobacterial phagosome immediately after phagosome formation; however this is followed by an increase in phagosomal pH within 24 hours (8, 25). The results of our study are consistent with a similar confocal microscopy study which demonstrated reduced colocalization of labeled *M. marinum*, a pathogen of fish and an opportunistic pathogen of humans, with acidified compartments (4).

Low levels of DAMP accumulation within the phagosomes containing live *M. a. ptb*, demonstrated by transmission electron microscopy indicates diminished acidification of these phagosomes, and supports the Lysotracker Red data generated by confocal microscopy (Figures 3, 4). DAMP is a congener of dinitrophenol (DNP) which becomes concentrated within acidic cellular compartments, and intensity of labeling of these compartments correlates to their acidity (1, 8). Although significantly reduced from phagosomes containing control samples or killed bacteria, accumulation of DAMP was not completely inhibited within the *M. a. ptb* containing phagosomes. It has been demonstrated for other species of pathogenic mycobacteria that while the phagosomal pH is reduced, it remains slightly lower than the cytosolic pH, and a small degree of acidification of the *M. a. ptb* phagosome may account for the limited positive DAMP staining (11).

By selectively inhibiting V-ATPase activity we show that, much like *M. avium* and *M. tuberculosis*, the method used by *M. a. ptb* to inhibit acidification is likely a lack of this proton pump within the surrounding phagosomal membrane. Although other vesicular transporters such as the Na^+/H^+ exchanger and Na^+/K^+ATPase have been proposed as having some role in phagosomal acidification, the V-ATPase proton pump has been shown to be the

main source of acidification (11, 14, 21). The V-ATPase proton pump is strongly inhibited by the microlide antibiotic Bafilomycin A_1 (14). Pretreatment of J774 cells with Bafilomycin A_1 lead to nearly complete loss of colocalization of Lysotracker Red with labeled *M. a. ptb*, *M. smegmatis*, and zymosan A, which shows the requirement of V-ATPase for acidification of mycobacterial and zymosan A phagosomes. These data suggest that exclusion of V-ATPase from the phagosomal membrane of *M. a. ptb* may be the mechanism used to inhibit phagosomal acidification. Our results are in agreement with additional studies that show exclusion of V-ATPase from the phagosomal membrane surrounding pathogenic mycobacteria as determined by inhibition with Bafilomycin A_1, and by detection of decreased V-ATPase on the mycobacterial phagosome by immunofluorescent analysis.

Our data demonstrate that viability of *M. a. ptb* is required in order for inhibition of phagosomal acidification to occur (Figure 1). Colocalization of Lysotracker Red with FITC labeled killed bacteria was markedly increased over that of live bacteria, more closely paralleling that of the zymosan A, indicating normal phagosomal acidification of killed *M. a. ptb* (Table 1). Consistent with the data from confocal studies, transmission electron microscopy evaluation of immunogold labeled DAMP within the phagosomal compartments of live and dead *M. a. ptb* demonstrated reduced acidification of the phagosomes of live bacteria, however killed bacteria were contained within acidified compartments. Our findings are consistent with other reports which show that viability is a requirement for inhibition of phagosomal acidification by pathogenic bacteria(4, 7, 16). These data suggest that the ability to inhibit acidification is a result of *M. a. ptb* actively manipulating the acidification of its phagosome, and this ability is lost following killing of the bacteria.

In the current study we also compared phagosomal acidification of *M. a. ptb* with *M. smegmatis*, and found increased acidification of the phagosomes containing *M. smegmatis* compared to those containing *M. a. ptb* (Table 1). These results are consistent with those of similar studies using Lysotracker Red and confocal microscopy which found increased acidification of phagosomes containing *M. smegmatis* compared to phagosomes containing *M. bovis* and *M. marinum* (23). *M. smegmatis* is considered to be a nonpathogenic species of mycobacteria; however, it is associated with opportunistic infections in several species of animals including humans(17, 19).

In this study, the number of acidified phagosomes containing *M. smegmatis* was significantly decreased from those containing zymosan A, and although no significant differences were noted between *M. smegmatis* and killed *M. a. ptb* ($P<.05$), there was a trend for phagosomal acidification of *M. smegmatis* to be intermediate between killed and live *M. a. ptb* for all time points (Table 1). These results suggest that at the time points evaluated in this study *M. smegmatis* may have some ability to resist phagosomal acidification. In contrast, in a similar report where infection times were extended, nearly all of the *M. smegmatis* was colocalized with Lysotracker Red by 72 hours postinfection (23). If infection times had been extended for longer periods in this study, a similar degree of acidification of the *M. smegmatis* phagosome may have been demonstrated.

The fact that the inocula contained dead *M. a. ptb* or *M. smegmatis* needs to be considered as these bacteria will colocalize with Lysotracker Red. This likely increased the percent colocalization of the live bacteria, especially *M. smegmatis* which had a larger percentage of nonviable bacteria in the inocula. Although not noted in this study, other reports have demonstrated large variability in mycobacterial phagosomal acidification at

earliest time points and this is speculated to be related to numbers of dead and injured bacteria in the original inocula (16).

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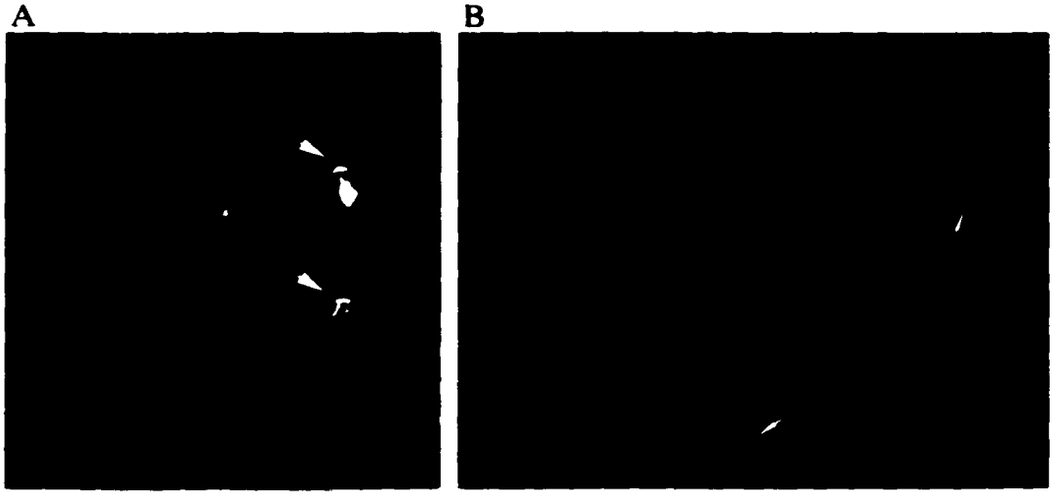
Table 1. Mean percent colocalization of bacteria with Lysotracker Red labeled compartments. Confocal microscopy was used to identify at least 50 random bacteria / zymosan A particles for each time point. The number of yellow (colocalized) bacteria were counted and the results expressed as a percentage of total (colocalized and noncolocalized) bacteria. Each value is the mean percentage of 3 replicates.

Agent	Time (hours)			Mean
	2	6	24	*
M. a. ptb	54.9	47.7	49.4	52.2^{A}
Killed *M. a. ptb*	78.7	82.4	76.0	79.3^{BC}
M smegmatis	67.4	76.4	65	69.7^{B}
Zymosan A	81.7	90.7	92	88.2^{C}

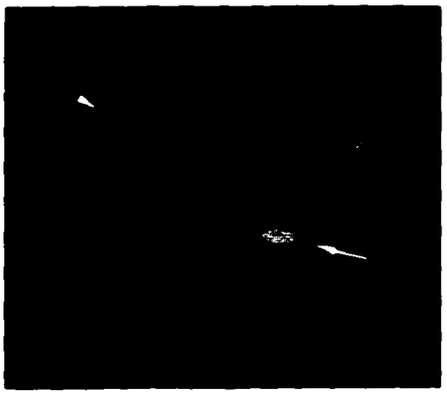
* indicates means within a column not sharing the same letter are statistically different ($P<0.05$). Within bacterial treatment groups no significant changes were noted over time.

figure1



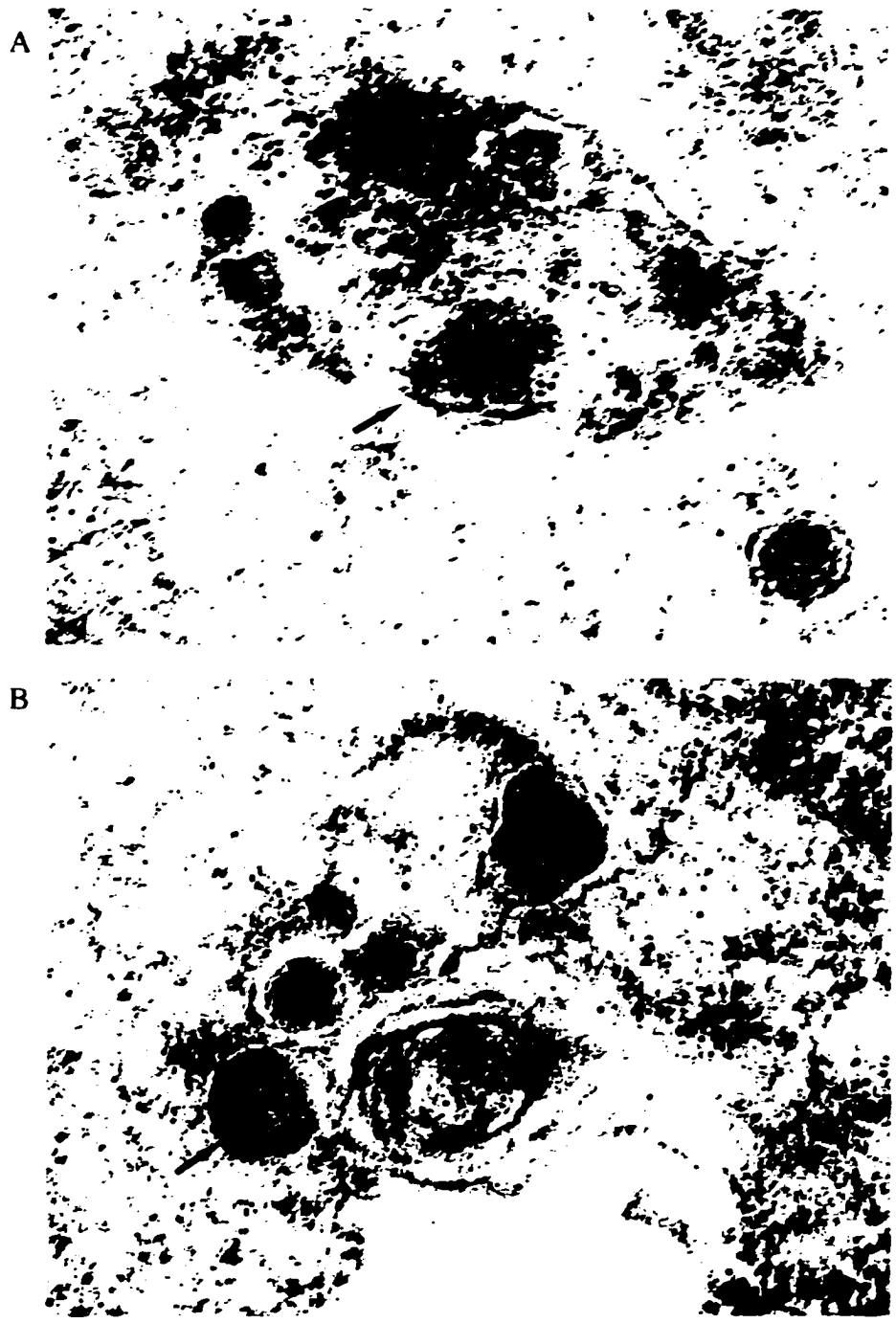
Colocalization of *M. a. ptb* with Lysotracker Red labeled compartments. At each time point Lysotracker Red was added to slides containing macrophages infected with either heat-killed (A), or live (B) *M. a. ptb* conjugated to FITC. Dead *M. a. ptb* (arrows) appear colocalized (yellow) indicating that they are within compartments of low pH (A), live *M. a. ptb* (arrows) appear noncolocalized (green) indicating that they reside within a compartment that is not acidified.

figure 2



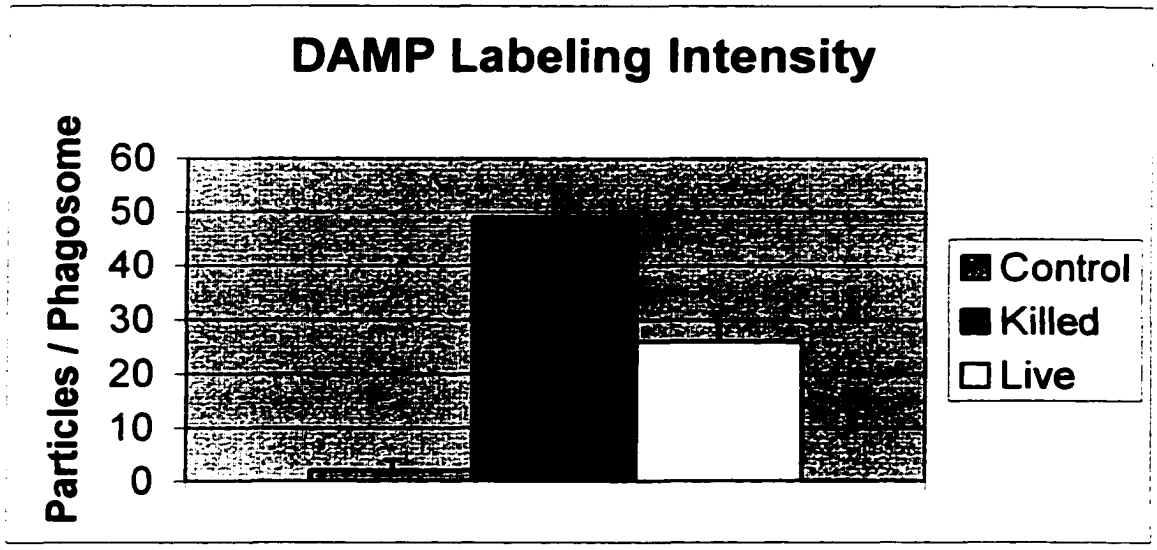
Inhibition of phagosomal acidification. Prior to infection with killed *M. a. ptb*, macrophages were incubated with the V-ATPase inhibitor Bafilomycin A_1. Following addition of Lysotracker Red, killed bacteria (arrow) remain green indicating that they are not located within acidified compartments (arrow head)

Figure 3



Immunogold DAMP labeling of phagosomes containing killed and live *M. a. ptb*: Immunogold DAMP labeling of killed (A) and live (B) *M. a. ptb* phagosomes at 2 hours. The number of gold particles is increased in the phagosomes of killed *M. a. ptb* (arrows) compared to those of live *M. a. ptb* (arrows). Decreased numbers of gold beads within the phagosomes of live *M. a. ptb* correlates with reduced acidification. Magnification 88,400X

Figure 4.



Intensity of DAMP labeling within *M. a. ptb* phagosomes. Immunogold labeling of DAMP within phagosomes containing live or killed *M. a. ptb*, or control sample (killed *M. a. ptb* without addition of DAMP) was evaluated by transmission electron microscopy. Gold particles were counted in at least 8 phagosomal compartments of similar size in 5-8 cells for each group, and results expressed as an average number of particles per phagosome. Killed *M. a. ptb* phagosomes contained increased numbers of gold labeled DAMP particles compared to live *M. a. ptb* indicating reduced acidification of the live *M. a. ptb* phagosomal compartments. Error bars indicate standard error of the means.

CHAPTER THREE. PHAGOSOMAL MATURATION AND INTRACELLULAR SURVIVAL OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN J774 CELLS

A paper prepared for submission to *Veterinary Pathology*

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Abstract

The mechanisms by which *Mycobacterium avium* subspecies *paratuberculosis* (*M. a. ptb*) survives within macrophages are not well characterized. One strategy for intracellular survival developed by *M. tuberculosis* and *M. avium* is inhibition of phagosomal maturation. It is our hypothesis that *M. a. ptb* is capable of survival within macrophages by residing within a phagosomal compartment that does not mature into a functional phagolysosome. To test this hypothesis the following objectives were determined. Phagosomal maturation was evaluated by comparison of stage specific markers on the membranes of phagosomes containing live *M. a. ptb* with killed *M. a. ptb*, *M. smegmatis*, or zymosan A using immunofluorescent labeling and confocal microscopy. Intracellular survival of live *M. a. ptb* within J774 macrophages was compared to that of *M. smegmatis* by direct determination of bacterial viability by differential live/dead staining. The results of this study show that the phagosomes containing *M. a. ptb* had increased levels of an early marker (transferrin receptor [TFR]) and decreased levels of a late maturation marker (lysosome associated membrane protein one [Lamp-1]), relative to killed *M. a. ptb, M. smegmatis*, or zymosan A. Additionally, compared to *M. smegmatis, M. a. ptb* has enhanced ability to survive within cultured macrophages. These data indicate that *M. a. ptb* resists intracellular killing by

residing within a phagosomal compartment that does not mature into a phagolysosome and retains the characteristics of early phagosomes.

Keywords: *Mycobacterium avium*, subspecies *paratuberculosis*, *Mycobacterium smegmatis*, transferrin receptor, lysosome associated membrane protein one, phagosomal maturation, J774 cells

Introduction

Johne's disease is a chronic progressive enteric disease of wild and domestic ruminants that is responsible for substantial economic losses to the livestock industry. Johne's disease is caused by *Mycobacterium avium*, subspecies *paratuberculosis* (*M. a. ptb*), a pathogenic species of mycobacteria which is an intracellular pathogen of monocytes and macrophages (28). A number of pathogens such as *Legionella pneumophila*, *Listeria monocytogenes*, and *Leishmania mexicana*, have developed mechanisms that enable them to survive within the potent antimicrobial environment of macrophages. These mechanisms include escape of the pathogen from the phagosome into the cytoplasm, inhibition of phagosomal acidification, and inhibition of phagosomal maturation (21).

Mechanisms used for intracellular survival have been described *M. tuberculosis, M. avium*, and other pathogenic mycobacteria; however, those used by *M. a. ptb* for intracellular survival are not well-characterized. One mechanism employed by pathogenic mycobacteria is inhibition of maturation of the phagosomal compartment in which they reside(1, 5, 20). By inhibiting maturation of phagosomes into phagolysosomes, pathogenic mycobacteria remain within a compartment that provides an environment that facilitates survival and replication.

During normal phagosomal maturation there is a series of stepwise interactions between the developing phagosome with early and late components of the endocytic pathway(11, 17). During these interactions, fusion and fission events occur between vesicles leading to sharing of membrane proteins and luminal contents between phagosomes and endosomes. In the early phases of phagosomal development phagosomes, acquire proteins associated with early endosomes while in the later phases of development, these proteins are lost and are replaced by proteins associated with late endosomes, then lysosomes(11, 12, 19). Over 200 different polypeptides have been isolated from early and late endosomes. A growing number of these polypeptides have been well-characterized and used as stage-specific markers of phagosomal maturation (10).

It is the hypothesis of this study that *M. a. ptb* survives within macrophages by inhibiting maturation of the phagosomal compartment in which it resides. To test this hypothesis we inoculated J774 cell monolayers with either live or killed *M. a. ptb*, *M. smegmatis*, or zymosan A and characterized phagosomal maturation by examining two stage specific markers, the transferrin receptor (TFR) and lysosome associated membrane protein 1 (Lamp-1), and examined their distribution by laser confocal microscopy.

Materials and Methods

Materials: Lab-Tek II chambered glass slides were purchased from Nalge Nunc International (Naperville, IL). The LIVE/DEAD® Bac*Light*™ kits were purchased from Molecular Probes Eugene, Oregon. Yeast wall extract Zymosan A and fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical Company (St. Louis, MO). Hybridoma cell lines producing monoclonal antibodies to lysosome associated membrane

protein one (LAMP- 1) (clone 104B) were purchased from The Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Hybridoma cell line 71B-219 producing monoclonal antibodies to the transferrin receptor (TFR) were purchased from American Type Culture Collection (ATTC). Cy-5 conjugated secondary antibodies were purchased from Zymed Laboratories (San Francisco, CA) . Vectasheild anti-fluorescent fading medium was obtained from Vector Laboratories (Burlingame, CA).

Bacterial Strains and Growth Conditions: Strain 19698 of *M a. ptb* was obtained from the USDA/ARS National Animal Disease Center (Ames, IA.). *Mycobacterium smegmatis* was obtained from ATCC (ATCC 607). Bacteria were grown in Middlebrook's 7H9 broth supplemented with Middlebrook's OADC enrichment and Mycobactin J. Bacteria were passed into fresh broth weekly to maintain log phase growth. Inoculum was prepared by sedimenting bacteria via centrifugation at 3000x *g* for 10 minutes, and then resuspending the pellet in physiologic saline. Passing the bacterial suspension through a 25-gauge needle repeatedly (at least 10X) immediately prior to inoculation of monolayers removed the majority of bacterial clumps. This led to approximately 85-90% single cell bacterial suspension. Bacterial numbers were determined by measuring absorbance of bacterial suspension at 540 nM and comparing values to graphical data of colony forming units of *M. a. ptb* vs absorbance at 540nM. Bacteria were killed by incubating at 80^0 C for 30 minutes.. Bacteria were killed by incubating at 80^0 C for 30 minutes.

Macrophage Cell Line: The mouse macrophage cell line J774A.1 (ATCC TIB-67) was maintained in a humidified, 37 °C, 5% CO_2, incubator. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 4 mM L-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate. Macrophages were

seeded onto chambered slides or 24 well tissue culture plates at 1.0 x 10^5 cells per ml and placed in the incubator overnight prior to infection.

Infection of Macrophages for Confocal Microscopy Studies: Mycobacteria and zymosan A were conjugated with FITC by a modification of a method described (2). Briefly, 4 x 10^7 broth grown bacteria or zymosan A were suspended in .001% FITC in a .2 M Na_2CO_3-$NaHCO_3$ buffer containing 150 mM NaCl (pH 9.2) for 30 minutes. Bacteria were washed twice in saline and resuspended in (DMEM). Bacteria were added to the monolayers of adherent J774 cells with a final multiplicity of infection (MOI) of 10:1.

Immunofluorescent Labeling: The method for immunofluorescent labeling of phagosome membrane markers was adapted from a method previously described (4). Briefly, monolayers were infected with *M. a. ptb* as described above. After 4 hours the first set of monolayers was processed for immunofluorescence. At this point the medium was removed from the remaining slides, and replaced with medium containing 0.1 mg/ml gentamycin for one hour followed by washing and replacement with fresh medium. Incubations were then continued for 24 and 48 hours. Following incubation, the monolayers were fixed for 1 hour in fresh Nakane's fixative (1% paraformaldehyde in 50 mM sodium phosphate buffer (pH 7.4), 10 mM sodium periodate, and 75 mM lysine). The monolayers were then permeabilized in buffer A, which consists of phosphate buffered saline with 0.1% gelatin, and 0.3% saponin. The cells were then incubated with the appropriate primary antibodies at 4°C overnight. The primary antibodies used were monoclonal antibodies Lamp–1 or the TFR each at a 1:2 dilution of their respective culture supernatants. Following overnight incubation, monolayers were washed with buffer A, and incubated with appropriate secondary antibodies conjugated to Cy-5 at a dilution of 1: 100 for 4 hours. After a final

wash in Buffer A, Vectasheild anti-fading mounting medium was added to the slides followed by coverslipping.

Laser Confocal Microscopy and Colocalization Studies: Images were generated and captured with a Leica TCS-NT confocal microscope (Leica Microsystems, Inc., Exton PA) equipped with krypton (568 nm-excitation wavelength) argon (488 nm-excitation wavelength) and HeNe (633 nm-excitation wavelength) lasers. The peak emissions for FITC and for Cy-5 are 518 nm and 670 nm respectively. These florigens were chosen due to the large difference between their wavelengths, which enhances their spectral separation by the confocal microscope. When signals from FITC labeled bacteria (green) overlapped with the Cy-5 signal from immunofluorescently labeled TFR or Lamp-1 (red), a yellow signal was generated (colocalization) (Figure 1). When the signals from labeled bacteria and phagosomal markers did not overlap, no shift in color was noted and these structures retained their original fluorescence (noncolocalized) (Figure 1). The method described here for counting colocalization of bacteria with phagosomal markers is a modification of a system developed for sampling intracellular bacteria by confocal microscopy (3). At each time point at least 50 individual bacteria/zymosan A were counted and images captured in at least 10 random fields for each slide. Images were then merged by IPlab (Scanalytics, Fairfax, VA.). Bacteria were then scored as being colocalized (yellow) or non-colocalized (green). Results were expressed as a percentage and were based on at least two replicates per experiment.

Intracellular Survivability –Live/Dead Assay: J774 cells were seeded onto 24 well tissue culture plates at a density of $1x10^5$ cells per well in 1 ml of media and incubated overnight at 37°C in 5% $C0_2$. After 24 hours, *M. a. ptb* or *M. smegmatis* were inoculated into the wells with a MOI of 10: 1. A 4 hour infection was followed by incubation of 24, 48,and

72, hours at 37°C. After the 4 hour time point, monolayers were washed 2 times with culture medium and medium containing gentamycin (.1 mg/ml) was replaced for 2 hours, followed by replacement with fresh media and continued incubation. At each time point monolayers were washed twice with phosphate buffered saline (PBS), bacteria were then harvested by lysing monolayers with 0.1% deoxycholate for 5 minutes. The resulting lysate was then incubated with Bac*Light* Live Dead kit for 15 minutes as recommended by the manufacturer. This staining method results in dead bacteria giving a red fluorescent signal and live bacteria a green signal. Live and dead bacteria were counted using an Olympus Bx 60 fluorescent microscope with the dual band (FITC/TRITC) filter set . For each chamber, 100 single bacterium were counted (counts done twice and the average of both counts reported) and each bacteria scored as either dead (red) or live (green). The results were expressed as a the percentage live bacteria. Results were based on triplicates of the experiments.

Statistical Analysis: Data were collected as percent colocalized for confocal studies and percent live for intracellular survival studies. Sources of variation were tested by General Linear Models procedure using SAS statistical software (SAS Institute, Cary, NC). Results were deemed significant at $P < 0.05$.

Results

Colocalization of *Mycobacterium avium* Subspecies *paratuberculosis* (*M. a. ptb)* with the Transferrin Receptor (TFR): There was increased colocalization of live *M. a. ptb* with labeled TFR relative to killed *M. a. ptb*, *M. smegmatis*, and zymosan A, (Table 1, Figure 1) ($P<.05$). The average value of colocalization for *M. a. ptb* at all time points was 25 %, which was significantly greater than that seen for killed *M. a. ptb* (7.2 %), *M. smegmatis*

(12.7 %), and zymosan A (3.5 %). No significant differences were noted for colocalization of live or dead *M. a. ptb*, *M. smegmatis*, or zymosan A with the TFR at 4, 24, or 48 hour time points. Zymosan A served as the positive control agent that would have normal maturation of its phagosomal compartment, and as expected, had a low degree of colocalization with the TFR. Killed *M. a. ptb* also had a low level of colocalization which was similar to that of zymosan A. The degree of colocalization of *M. smegmatis* was similar to that of killed *M. a. ptb*, but was significantly increased over that of zymosan A.

Colocalization of *M. a. ptb* with Lysosome Associated Membrane Protein One (Lamp-1): There was reduced colocalization of *M. a. ptb* with Lamp-1 compared to that of killed *M. a. ptb, M. smegmatis*, and zymosan A (Table 2, Figure 2). The average percent colocalization for *M. a. ptb* with the Lamp-1 marker for all time points was 61.9 %, which is significantly lower than the averages of all time points for killed *M. a. ptb* (81.4 %), *M. smegmatis* (75.0 %) and zymosan A (87.0 %). Similar to what was seen for the TFR, no significant changes in colocalization were detected at the three time points for any of the agents tested. As expected, the positive control, zymosan A, had a high degree of colocalization with Lamp-1. Killed *M. a. ptb* also had a high degree of colocalization with Lamp-1, which was similar to that of zymosan A. Colocalization of *M. smegmatis* with Lamp-1 was slightly decreased from that of killed *M. a. ptb* and zymosan A.

Intracellular Survivability - BacLight Staining System: Increased percentages of live *M. a. ptb* were identified compared to live *M. smegmatis* (Figure 3). The average percent of live *M. a. ptb* for all time points was 72.8 % compared to 53.4 % for *M. smegmatis*. During the course of the experiment, the percentages live *M. a. ptb* was maintained at similar values; however, there was marked decrease in percentage live *M. smegmatis* between 4 and

24 hour time points. Initially, the percent survival was similar for *M. a. ptb* and *M. smegmatis* with 76.4 % and 72.4 % live at the 4 hour time point. By the 24 hour time point percent live for *M. a. ptb* was 70.3 % while that of *M. smegmatis* had dropped to 48.9 %. The percent live values for *M. a. ptb* and *M. smegmatis* remained close to these values for the remaining time points (Figure 3).

Discussion

The results of this study demonstrate that *M. a. ptb* is able to survive intracellularly within macrophages and that there is diminished ability of the phagosome containing *M. a. ptb* to obtain markers associated with normal phagosomal maturation. Thus, like other pathogenic species of mycobacteria, inhibition of phagosomal maturation appears to be an important virulence factor for *M. a. ptb*. It has been demonstrated for *M. avium* and *M. tuberculosis* that, by remaining fusogenic with early phagosomal/endosomal compartments, these phagosomes do not interact with late endosomes or lysosomes, therefore do not develop into functional phagolysosomes(5, 8). By residing within phagosomes that do not undergo phagolysosome development, *M. a. ptb* resists degradation by remaining outside the antimicrobial environment of the phagolysosome, which includes acid hydrolases, proteases, and acidic pH, and additionally avoids antigenic processing thereby remaining sequestered from adaptive immune responses(14, 22).

Increased colocalization of *M. a. ptb* with the transferrin receptor (TFR), demonstrated by confocal microscopy, indicates that *M. a. ptb* resides within a phagosomal compartment that retains features of an early phagosomal/endosomal compartment (Figure1). The TFR binds iron-bound transferrin at the cell surface and following ligation, the TFR is

internalized into coated vesicles that fuse with early endosomes. Following acidification of the endosome, iron is released from transferrin, and the TFR and transferrin recycle to the cell surface, thus the TFR does not traffic through the late endosomal and lysosomal compartments making it a marker of early endosomes and phagosomes(7, 16). Increased colocalization of *M. a. ptb* with the TFR indicates enrichment of the *M. a. ptb* phagosomal membrane with this receptor. Although the majority of *M. a. ptb* did not colocalize with the TFR, the percent that did colocalize was significantly higher than that of other agents tested, and these data are consistent with that of another study evaluating presence of the TFR within phagosomal membranes of *M. avium* (25). Colocalization of killed *M. a. ptb* was markedly reduced from live *M. a. ptb* indicating that phagosomes of killed bacteria are no longer fusogenic with early endosomal compartments and thus have lost the TFR from their phagosomal membrane.

The presence of TFR within the phagosomal membranes of *M. a. ptb* indicates continued communication of these phagosomes with early endosomal compartments. Potential sources of the TFR are the biosynthetic pathway, the plasma membrane, and early endosomes. It has been demonstrated that following exogenous administration of transferrin to *M. tuberculosis* infected macrophages, the phagosome containing *M. tuberculosis* became enriched with the exogenous transferrin indicating that the source of transferrin and its receptor was delivery from early endosomes (6). In this study, it is likely that like the phagosome containing *M. tuberculosis*, the source of the TFR within the phagosome containing *M. a. ptb* membrane is delivery from early endosomes. This suggests that the phagosome containing *M. a. ptb* continues to fuse with early endosomes maintaining a phagosomal membrane composition similar to early endosomal compartments.

Decreased colocalization of the *M. a. ptb* with Lamp-1 demonstrates that phagosomes containing *M. a. ptb* fail to mature into the phagolysosome stages (Figure 2). The function of Lamp-1 is poorly understood; however, it may have a protective role within lysosomal membranes (13). Lamp-1 is present in highest concentrations within late endosomal and lysosomal compartments, and its concentration has been shown to increase within the phagosomal membranes during progression from early to late phagosome and phagolysosomal stages (24). Although colocalization of *M. a. ptb* with Lamp-1 was not abolished, it was significantly decreased compared to the other agents tested, which indicates reduced acquisition of this marker by the phagosomal compartments containing *M. a. ptb*. There is some contrast in reports regarding Lamp-1 concentrations within the phagosomes containing mycobacteria. Some indicate that Lamp-1 is actually enriched within mycobacterial phagosomes and may be reaching these phagosomes by alternate trafficking pathways(9, 26, 27). In contrast, several studies show low to intermediate levels of Lamp-1 within mycobacterial phagosomes, and the results of the present study are consistent with one such study reporting reduced levels of Lamp-1 accumulation within phagosomal compartments containing live *M. marinum* compared to those containing killed organisms using confocal microscopy(3, 5, 15). As phagosomal compartments typically acquire Lamp-1 by interaction with late endosomal and lysosomal compartments, the reduced concentration of Lamp-1 within the phagosomal membrane indicates restricted interaction between the *M. a. ptb* phagosome and late endocytic and lysosomal compartments, and thus failure of maturation into these late phagosomal stages.

Mycobacterium avium subspecies *paratuberculosis* needs to be viable in order to prevent phagosomal maturation. Following heat killing of *M. a. ptb*, there was nearly a

complete reversal of the trends demonstrated for live *M. a. ptb* regarding colocalization with the TFR and Lamp-1. Colocalization events for killed *M. a. ptb* and the TFR dropped to a level similar to the control agent zymosan A, while those for Lamp-1 increased to a level similar to zymosan A. These results suggest that some feature of live *M. a. ptb* or factors produced by the organism influence phagosomal maturation.

When the ability to inhibit phagosomal maturation of *M. a. ptb* was compared to *M. smegmatis*, there was a marked increase in the TFR and decreased Lamp-1 colocalization in the phagosomes containing *M. a. ptb* compared to those of the *M. smegmatis*. This indicates that compared to *M. a. ptb*, there is increased ability of the phagosome containing *M. smegmatis* to maturation into a phagolysosome. It is interesting to note that there were significant differences in percent colocalization of the TFR between *M. smegmatis* and killed *M. a. ptb* and between *M. smegmatis* and the control agent zymosan A, and Lamp-1 followed a similar pattern, however differences between *M. smegmatis* and killed *M. a. ptb* were not statistically significant. There was increased TFR and decreased Lamp-1 colocalization within the phagosome containing *M. smegmatis* compared those containing zymosan A, and the level of colocalization with these markers tended to be intermediate between phagosomes containing live and killed *M. a. ptb*. These results suggest that there may be some ability, although markedly reduced from that of *M. a. ptb*, of *M. smegmatis* to influence phagosomal maturation. Some ability to interfere with phagosomal processing may act as a virulence factor for this organism. While *M. smegmatis* is reported as a nonpathogenic species of mycobacteria, it is an opportunistic pathogen, and with the right conditions can cause disease in animals and man(18, 23).

There was a marked increase in percent survival of *M. a. ptb* within J774 cells over that of *M. smegmatis* (Figure 3). In contrast to the data obtained for phagosomal markers where time did not appear to be a factor in phagosomal membrane composition, death of *M. smegmatis* was not detectable until 24 hours after infection. The Bac*Light* system, used in this study, is capable of differentiating live from dead bacteria by differential uptake of stains by viable and nonviable bacterial cell walls. A disadvantage of this system is that it gives only percentages rather than an absolute number of live and dead bacteria. A strong advantage of this system when used for determining mycobacterial survivability is that only single bacteria are counted, which in the colony forming assay (CFU) is not always the case. The tendency of mycobacteria to clump means that a certain percentage of colonies that grow up are derived from a clump containing multiple bacteria, rather than being derived from a single bacterium which may contribute to error when calculating the final number of live bacteria . In some studies these techniques have been combined in order to get a more accurate number of live bacteria (3). Another advantage of using the Bac*Light* system is the greatly reduced amount of time required to obtain results over the traditional CFU assays.

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Table 1. Mean percent colocalization of bacteria with the transferrin receptor. Confocal microscopy was used to identify at least 50 random bacteria or zymosan A at each time point. The number of yellow (colocalized) bacteria were counted and the results expressed as a percentage of total (colocalized and noncolocalized) bacteria. Each value is the mean percentage of 3 replicates.

Agent	Time (hours)			Mean
	4	24	48	*
M. a. ptb	26.0	29.7	20.3	25.3[A]
Killed *M. a. ptb*	7.3	8.7	5.7	7.2[BD]
M. smegmatis	17.0	9.3	11.7	12.7[C]
Zymosan A	2.7	3.0	4.7	3.5[D]

* indicates means within a column not sharing the same letter are statistically different. ($P<0.05$). Within bacterial treatment groups no significant changes were noted over time.

Table 2. Mean percent colocalization of bacteria with Lamp-1. Confocal microscopy was used to identify at least 50 random bacteria or zymosan A at each time point. The number of yellow (colocalized) bacteria were counted and the results expressed as a percentage of total (colocalized and noncolocalized) bacteria. Each value is the mean percentage of 3 replicates.

Agent	Time (hours)			Mean
	4	24	48	*
M. a. ptb	63.0	65.7	52.0	61.9^{A}
Killed *M. a. ptb*	85.0	77.7	82.3	81.4^{BC}
M smegmatis	75.0	82.5	67.7	75.0^{B}
Zymosan A	92.3	87.0	81.7	87.0^{C}

* indicates means within a column not sharing the same letter are statistically different. ($P<0.05$). Within bacterial treatment groups no significant changes were noted over time.

figure1

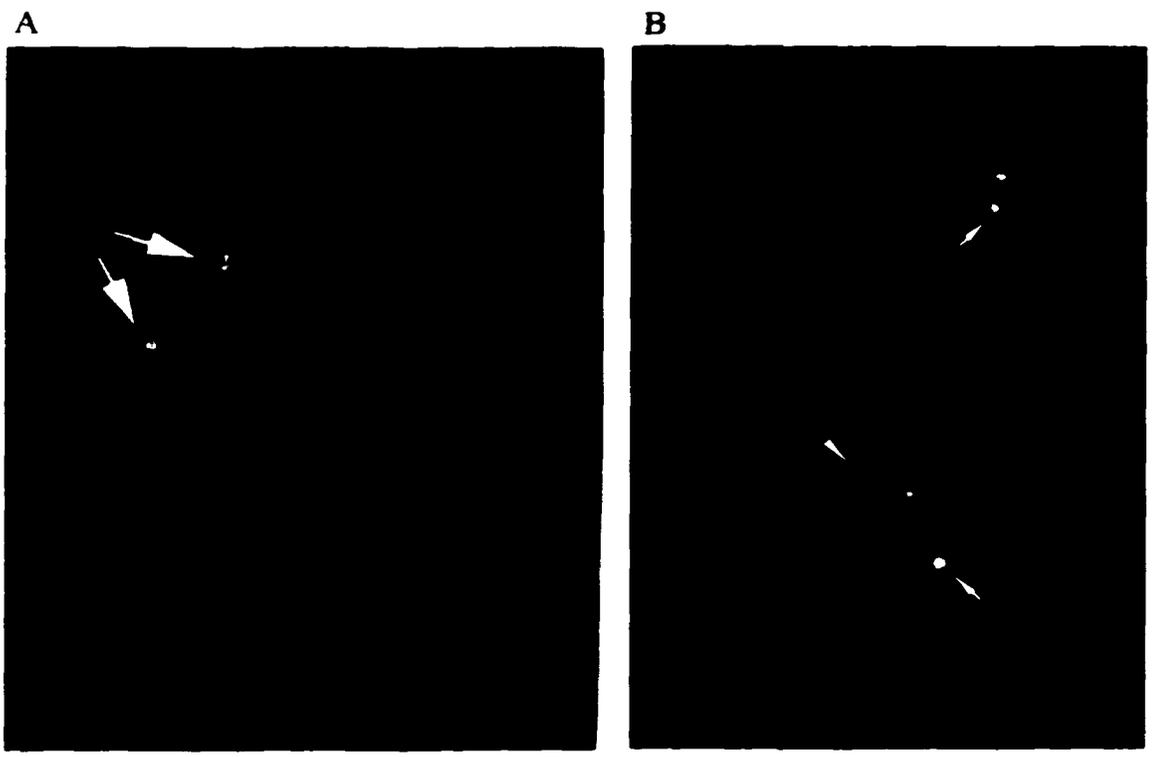


Figure 1.
Colocalization of *M. a. ptb* with TFR labeled compartments. Macrophages were infected with either killed (A) or live *M. a. ptb* (B) conjugated to FITC. Following each time point the TFR was immunofluorescently labeled. Dead *M. a. ptb* appear noncolocalized (green) (arrow) indicating their phagosomes have progressed to a stage which no longer communicates with early endosomes and have subsequently lost the TFR from their membrane (A). Live *M. a. ptb* (B) appear colocalized (yellow) indicating that these phagosomal compartment are enriched with the TFR (arrows) and have retained features of an early phagosomal compartments. Live *M. a. ptb* are also present which appear green (arrowhead) demonstrating that not all live organisms were able to inhibit phagosome development.

figure 2

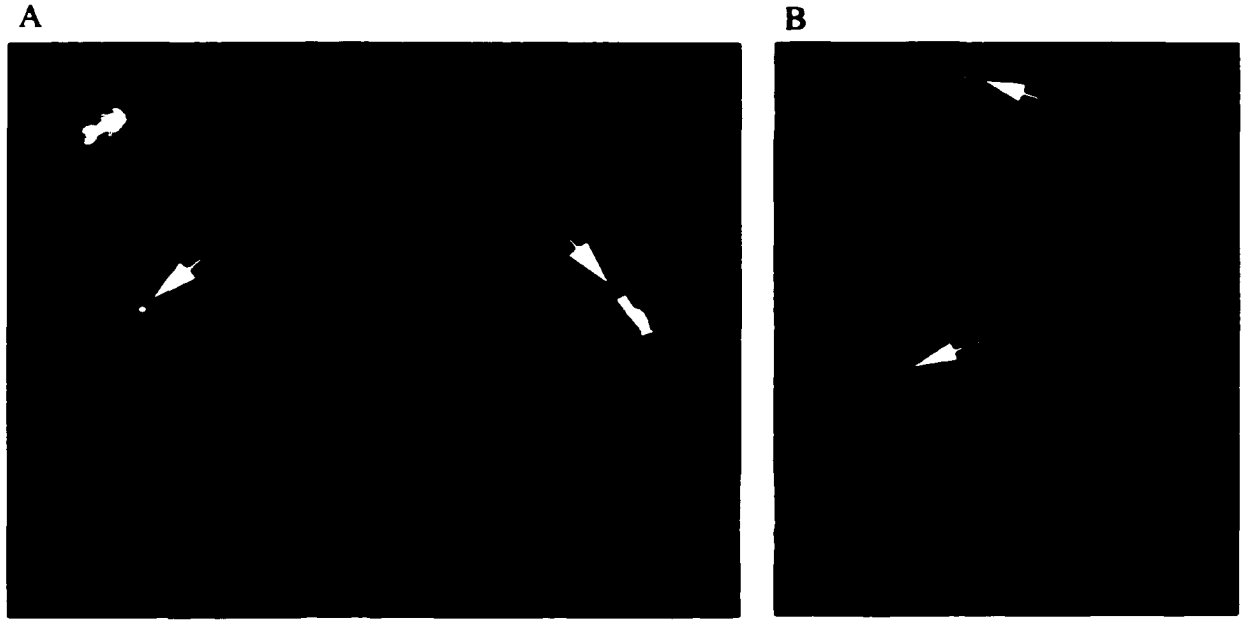
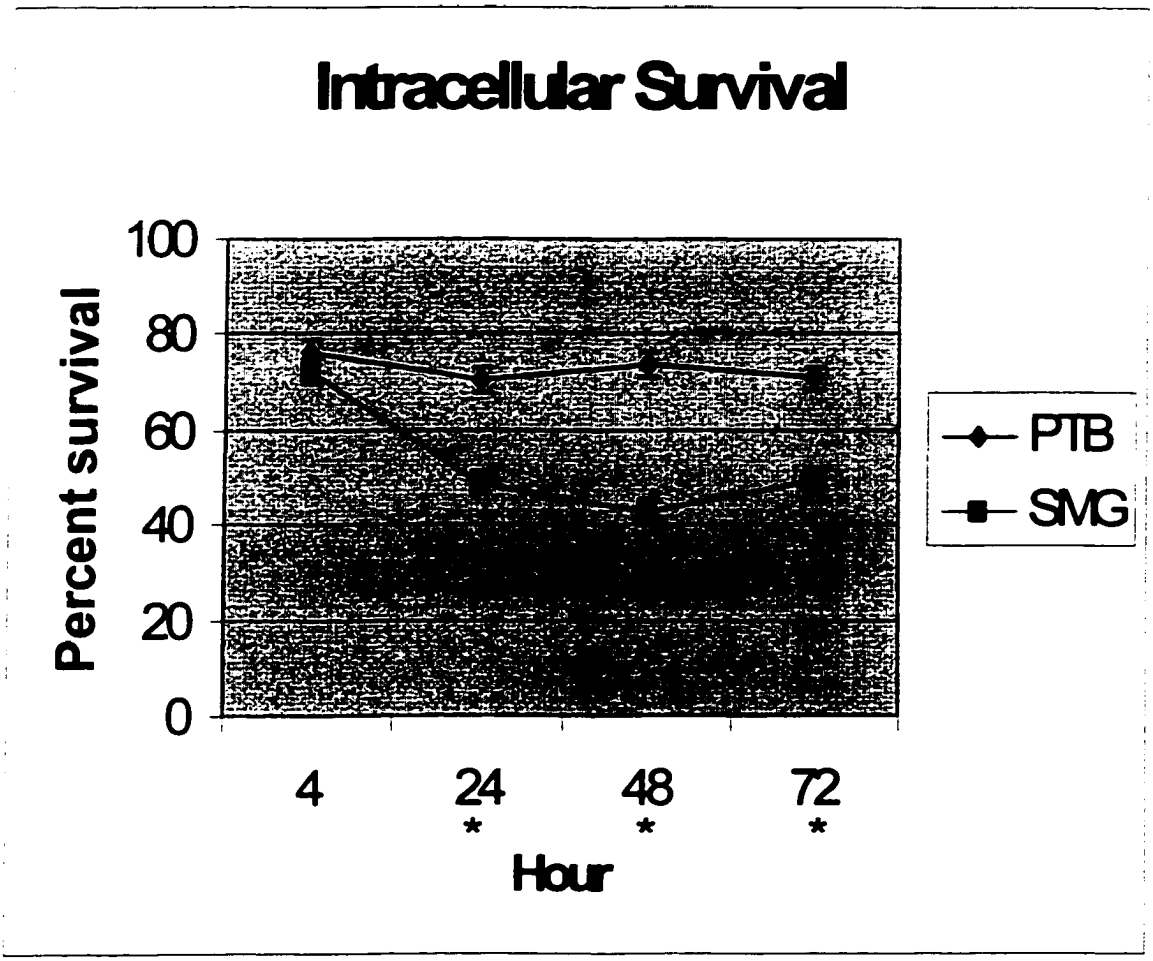


Figure 2. Colocalization of bacteria with Lamp-1. Macrophages were infected with either killed (A) or live *M. a. ptb* (B) conjugated to FITC. Following each time point Lamp-1 was immunofluorescently labeled. Dead *M. a. ptb* appear colocalized (yellow) (arrow) indicating they reside within a phagosome which is enriched with the Lamp-1 marker and thus have matured into the late phagosome/phagolysosome stages (A), while live *M. a. ptb* (B) appear noncolocalized (green) indicating that their phagosomal compartment have reduced Lamp-1 and have reduced ability to mature into late stages (arrows).

Figure 3



Intracellular survival of *M. a. ptb* and *M. smegmatis*. Macrophages were infected with either *M. a. ptb* or *M. smegmatis*. Following each time point macrophages were lysed and viability of the released bacteria was determined by the BacLight staining system. Results are the mean percent survival values from 3 replicates and indicate significantly increased survival of *M. a. ptb* from the 24 hour time point on. Asterisk at each time point indicates means are significantly different ($P<0.05$) from *M. smegmatis* infected cultures

CHAPTER FOUR. CYTOKINE EFFECTS ON MATURATION OF THE PHAGOSOMES CONTAINING *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN J774 CELLS

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A paper to be submitted to *Veterinary Pathology*

Abstract

Mycobacterium avium subspecies *paratuberculosis* (*M. a. ptb*) is an intracellular pathogen of monocytes and monocyte derived macrophages. It has been demonstrated that there is decreased ability of *M. tuberculosis* and *M. avium* to inhibit phagosomal maturation in cytokine treated macrophages. It is the hypothesis of this study that there is increased acidification and maturation of the *M. a. ptb* phagosome in interferon gamma and lipopolysaccharide (IFN-γ /LPS) treated macrophages. To test this hypothesis, IFN-γ/LPS treated macrophages were infected with fluorescein isothiocyanate (FITC) labeled *M. a. ptb*, and colocalization of *M. a. ptb* with either Lysotracker Red labeled acidic compartments or immunofluorescently labeled lysosome associated membrane protein 1 (Lamp-1) was evaluated by laser confocal microscopy. Additionally, percent intracellular survival of *M. a. ptb* was evaluated by direct determination of bacterial viability using differential live/dead staining following infection of IFN-γ/LPS treated macrophages. The results of this study demonstrated increased colocalization of both Lysotracker Red and Lamp-1 with FITC labeled *M. a. ptb*, and decreased percentages of live *M. a. ptb* within IFN-γ/LPS treated macrophages. These results indicate that IFN-γ/LPS treated J774 cells have increased ability

to cause maturation of the phagosome containing *M. a. ptb* into a functional phagolysosome compartment.

Key words: *Mycobacterium avium* subspecies *paratuberculosis*, cytokines, phagosomal acidification, phagosomal maturation, Lamp-1, Lysotracker Red

Introduction

Mycobacterium avium subspecies *paratuberculosis* (*M. a. ptb)* an intracellular pathogen of monocytes and macrophages, and is the causative agent of Johne's disease, which is a chronic progressive enteritis of domestic and wild ruminants. *M. a. ptb* has been classified as a subspecies of *M. avium* due to the high degree of homology of the genomes between these two organisms(6, 13, 14). *M. avium* and other pathogenic species of mycobacteria, including *M. tuberculosis*, have evolved mechanisms which enhance their ability to survive intracellularly within macrophages. Following phagocytosis, these bacteria reside within a phagosomal compartment which has reduced ability to become acidified and does not undergo normal phagosomal maturation into a functional phagolysosome, thereby creating a protective environment which facilitates bacterial survival and replication(7, 18, 20). Results from two reports currently in progress by these authors suggest that, like closely related *M. avium*, *M. a. ptb* also appears to be capable interfering with the normal development of its phagosomal compartment(11, 12).

Cytokine activation of macrophages plays an important role in control and elimination of many intracellular pathogens. Cytokines such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin 4 (IL-4), and granulocyte-macrophage

colony stimulating factor (GM-CSF), either alone or in combination, have been shown to increase the capability of macrophages to control mycobacterial infections both *in vitro* and *in vivo*(10, 16, 19). Multiple mechanisms are likely involved which allow activated macrophages to restrict intracellular survival and growth of mycobacteria including enhanced production of nitrogen and oxygen intermediates(10, 16, 19). In addition to production of reactive intermediates, cytokine treatment of macrophages has been shown to increase acidification and maturation of phagosomal compartments containing *M. avium*(2, 17).

It is the hypothesis of this study that there is decreased ability of *M. a. ptb* to inhibit acidification and maturation of its phagosomal compartment in interferon gamma and lipopolysaccharide (IFN-γ /LPS) treated macrophages. To test this hypothesis we inoculated either live or heat killed *M. a. ptb* into non-treated and IFN-γ /LPS treated macrophages, and compared acidification and maturation of their phagosomal compartments by the addition of the acidotrophic marker Lysotracker Red and immunofluorescent labeling of the late phagosome stage specific protein lysosome associated membrane protein 1 (Lamp-1). Colocalization of these markers with fluorescein isothiocyanate (FITC) labeled bacteria was accomplished by laser confocal microscopy. We selected IFN-γ /LPS treatment based on reports indicating a synergistic effect of IFN-γ /LPS on activation of macrophages and on data reported in similar *in vitro* studies with pathogenic mycobacteria(1, 17, 19).

Materials and Methods

Materials: Lab-Tek II chambered glass slides were purchased from Nalge Nunc International (Naperville, IL). Lysotracker Red ™ and LIVE/DEAD® Bac*Light*™ kits were purchased from Molecular Probes(Eugene, Oregon). Fluorescein isothiocyanate (FITC),

mouse recombinant interferon gamma (IFN-γ), and E. coli (0111:B4) LPS were purchased from Sigma (St. Louis, MO). The hybridoma cell line (clone 104B) producing monoclonal antibodies to lysosome associated membrane protein 1 (Lamp-1) was purchased from The Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Cy-5 conjugated secondary antibodies were purchased from Zymed Laboratories (San Francisco, CA) . Vectasheild anti-fluorescent fading medium was obtained from Vector Laboratories (Burlingame, CA).

Bacterial Strains and Growth Conditions: The standard American Type Culture Collection (ATCC) strain of *Mycobacterium avium* subspecies *paratuberculosis* (*M a. ptb)* 19698, was obtained from the USDA/ARS National Animal Disease Center (Ames, IA.). *M. smegmatis* was obtained from ATCC (ATCC 607). Bacteria were grown in Middlebrook's 7H9 broth supplemented with Middlebrook's OADC enrichment and Mycobactin J. Bacteria were passed into fresh broth weekly to maintain log phase growth. Inoculum was prepared by sedimenting bacteria via centrifugation at 3000x *g* for 10 minutes, and then resuspending the pellet in sterile physiological saline. Passing the bacterial suspension through a 25-gauge needle repeatedly (at least 10X) immediately prior to inoculation of monolayers eliminated bacterial aggregates. This led to approximately 85-90% single cell bacterial preparation. Bacterial numbers were determined by measuring absorbance of bacterial suspension at 540 nM and comparing values to graphical data of colony forming units of *M. a. ptb* vs. absorbance at 540 nM..

Macrophage Cell Line: The mouse macrophage cell line J774A.1 (ATCC TIB-67) was maintained in a humidified, 37 °C, 5% CO_2 incubator. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 4 mM L-

glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate. Prior to addition of bacteria, macrophages were seeded onto chambered slides or 24 well tissue culture plates at 1.0×10^5 cells per ml and incubated overnight at 37°C in an atmosphere of 5 % $C0_2$.

Cytokine Treatment: The protocol for treating macrophages with IFN-γ and LPS was adapted from a method previously described (19). Briefly, DMEM containing 500 units/ml of IFN-γ and 1ug/ml of LPS was added to monolayers of J774 cells and incubated for 24 hours in a humidified 37°C incubator with 5% $C0_2$. Concentrations of IFN-γ and LPS were then maintained in the culture medium during and after infection periods.

Infection of Macrophages for Confocal Microscopy Studies: Mycobacteria were conjugated with FITC by a modification of a method previously described (3): 4×10^7 broth grown bacteria were suspended in .001% FITC in a .2 M Na_2CO_3-$NaHCO_3$ buffer containing 150mM NaCl (pH 9.2) for 30 minutes. Bacteria were then washed twice in saline and resuspended in DMEM. Labeled bacteria were added to monolayers of both non-treated and IFN-γ/LPS treated macrophages with a final multiplicity of infection (MOI) of 10:1.

Lysotracker Red: For Lysotracker Red studies bacteria were incubated with non-treated and IFN-γ/LPS treated macrophages for 2, 6, 24, and 48 hour periods. DMEM containing 50 nM Lysotracker Red was added to the chambered slides during the last 60 minutes of each incubation. Following incubation, monolayers were washed with two changes of medium (wash medium contained 50 nM Lysotracker Red) to remove extracellular bacteria. Following the last wash slides were coverslipped and evaluated immediately following preparation to minimize the fading effects of Lysotracker Red.

Immunofluorescent Labeling: The method for immunofluorescent labeling of phagosome membrane markers was adapted from a method previously described (5). Briefly,

non-treated and IFN-γ /LPS macrophages were infected with *M. a. ptb* as described above. After 4 hours, the first set of monolayers were processed for immunofluorescence. At this point the medium was removed from the remaining slides, and replaced with medium containing 0.1 mg/ml gentamycin for one hour followed by washing and replacement with fresh media. Incubations were then continued for 24 and 48 hours. Following incubation, the monolayers were fixed for 1 hour in fresh Nakane's fixative (1% paraformaldehyde in 50mM sodium phosphate buffer (pH 7.4), 10mM sodium periodate, and 75 mM lysine). The monolayers were then permeabilized in buffer A, which consists of phosphate buffered saline with 0.1% gelatin, and 0.3% saponin. The cells were then incubated with the primary antibody at 4°C overnight. The primary antibody was monoclonal antibody against Lamp–1 at a 1:2 dilution of clone IDB culture supernatant. Following overnight incubation, monolayers were washed with buffer A, and incubated with secondary antibody conjugated to Cy-5 at a dilution of 1: 100 for 4 hours. After a final wash in Buffer A, Vectasheild anti-fading mounting medium was added to the slides followed by coverslipping.

Laser Confocal Microscopy and Colocalization Studies: Images were generated and captured with a Leica TCS-NT confocal microscope (Leica Microsystems, Inc., Exton PA) equipped with krypton (568 nm-excitation wavelength) argon (488 nm-excitation wavelength) and HeNe (633 nm-excitation wavelength) lasers. The peak emissions for FITC and for Cy-5 are 518 nm and 670 nm respectively. The peak emmission for Lysotracker Red is 592 nm. The differences in the emmission wavelengths of FITC and Cy-5, and FITC and Lysotracker Red were adequate for spectral separation of these signals by the confocal microscope. When signals from FITC labeled bacteria (green) overlapped with Lysotracker Red (red) or the Cy-5 signal from immunofluorescently labeled Lamp-1 (red), a yellow

signal was generated (colocalization). When the signals from labeled bacteria and phagosomal markers did not overlap, no shift in color was noted and these structures retained their original fluorescence (noncolocalized). The method described here for counting colocalization of bacteria with phagosomal markers is a modification of a system developed for sampling intracellular bacteria by confocal microscopy (4). At each time point at least 50 individual bacteria were counted and images captured in at least 10 random fields for each slide. Images were then merged by IPlab (Scanalytics, Fairfax, VA.). Bacteria were then scored as being colocalized (yellow) or non-colocalized (green). Results were expressed as a percentage and were based on at least two replicates per experiment.

Intracellular Survivability – Live/Dead Assay: J774 cells were seeded into two separate 24 well tissue culture plates at a density of $1x10^5$ cells per well in 1 ml of media and placed overnight in an incubator at 37°C with 5% $C0_2$. Monolayers in one set of wells were incubated with DMEM, while the second was incubated with DMEM containing IFN-γ/LPS (described above). After 24 hours *M. a. ptb* or *M. smegmatis* were inoculated into the wells of non-treated and IFN-γ/LPS treated macrophages with a MOI of 10: 1. A 4 hour infection was followed by incubation of 24, 48,and 72, hours at 37°C. After the 4 hour infection, monolayers were washed 2 times with culture medium and medium containing gentamycin (.1 mg/ml) was replaced for 2 hours, followed by replacement with fresh medium and continued incubation. At the each time point, monolayers were washed twice with PBS and bacteria were then harvested by lysing monolayers with 0.1 % deoxycholate for 5 minutes. The resulting lysate was incubated with Bac*Light* Live/Dead kit for 15 minutes as recommended by the manufacturer. This staining method results in dead bacteria giving a red fluorescent signal and live bacteria a green signal. Live and dead bacteria were counted using

an Olympus Bx 60 fluorescent microscope with the dual band (FITC/TRITC) filter set . For each chamber, 100 single bacteria were counted (counts done twice and the average of both counts reported) and each bacterium scored as either dead (red) or live (green). The number of live bacteria was expressed as a percentage of total (live and dead) bacteria. Results were based on six replicates of the experiment.

Statistical Analysis: Data were collected as percent colocalized for confocal studies and percent live for intracellular survival studies. Sources of variation were tested by General Linear Models procedure using SAS statistical software (SAS Institute, Cary, NC). Results were deemed significant at $P < 0.05$.

Results

Colocalization of *M. a. ptb* with Lysotracker Red in IFN-γ/LPS Treated Macrophages: There was increased colocalization of *M. a. ptb* with Lysotracker Red in macrophages treated with IFN-γ and LPS relative to that of non-treated macrophages (Table 1, Figure 1). The average value for percent colocalization of *M. a. ptb* with Lysotracker Red for all time points was 75.5 % in IFN-γ/LPS treated macrophages, which is significantly increased from that of non-treated macrophages which was 52.9 % ($P<0.5$). The average values for percent colocalization between killed *M. a. ptb* and Lysotracker Red were very similar for both IFN-γ/LPS treated (87.5 %) and non-treated macrophages (86.1 %). Time did not have a significant affect on bacterial colocalization with Lysotracker Red in treated or non-treated macrophages. While the colocalization values were significantly different between live and killed *M. a. ptb* in non-treated macrophages (52.9% and 87.5% respectively), there were no significant differences ($P<.05$) between live *M. a. ptb* in IFN-

γ/LPS treated macrophages and killed *M. a. ptb* in either non-treated or IFN-γ/LPS treated macrophages (75.5% for *M. a. ptb* and 87.5% for killed *M. a. ptb* in non-treated macrophages, and 86.1% in IFN-γ/LPS treated macrophages).

Colocalization of *M. a. ptb* with Lamp-1 in IFN-γ/LPS Treated Macrophages: There was increased colocalization of FITC labeled *M. a. ptb* with the late phagosome marker Lamp-1 in IFN-γ/LPS treated macrophages compared to that of non-treated macrophages (Table 2, Figure 2). The mean percent colocalization value of *M. a. ptb* with Lamp-1 for all time points in IFN-γ/LPS treated macrophages was 68.3%, which is significantly increased from 39.0 %, the mean value for percent colocalization in non-treated macrophages ($P<.05$). Similar to the Lysotracker Red studies, time did not have a significant effect on colocalization of bacteria with Lamp-1 in treated or non-treated macrophages. Mean percent colocalization of live *M. a. ptb* with Lamp-1 was significantly lower than that of killed *M. a. ptb* (39.0 % and 75.6 %, respectively). In non treated macrophages; however, these values were no longer significantly different in IFN-γ/LPS treated macrophages (68.3 % for live *M. a. ptb* and 74.4 % for killed *M. a. ptb*). There were no significant differences between mean percent colocalization of killed *M. a. ptb* in non treated and IFN-γ/LPS treated macrophages (75.6% and 74.4% respectively).

Intracellular Survival of *M. a. ptb* within IFN-γ/LPS Treated J774 Cells: There was a significant difference in percent survival of *M. a. ptb* within IFN-γ/LPS treated and non-treated macrophages (Table 3). There were no significant differences in the percent survival between *M. a. ptb* and *M. smegmatis* during infection periods, which is in contrast to data from previous report by the authors of this manuscript (12). There was a trend; however, for the percent survival of *M. smegmatis* to remain below that of *M. a. ptb*, especially

following the 4 hour time point in this study. In non-treated macrophages, there were no significant changes in percent survival of *M. a. ptb* among infection periods. In IFN-γ/LPS treated macrophages the percent survival of *M. a. ptb* was significantly reduced from percent survival of *M. a. ptb* in non treated macrophages, and percent survival of *M. smegmatis* in treated and non-treated macrophages. The reduced survival of *M. a. ptb* in IFN-γ/LPS was significant following the 4 hour infection period.

Discussion

The results of this study support the hypothesis that treatment of J774 macrophages with IFN-γ and LPS leads to a shift towards increased maturation of the phagosomes containing *M. a. ptb*. Inhibition of phagosomal acidification and maturation, described in two reports in progress by these authors, appears to be important mechanisms used for intracellular survival by *M. a. ptb*(11, 12). The data in this study indicate that there is reduced ability for the *M. a. ptb* to influence phagosomal development in macrophages treated with IFN-γ and LPS. With increased ability of the treated macrophage to initiate normal development of the phagosome containing *M. a. ptb*, the protective environment in phagosomes containing *M. a. ptb* is not attained, but rather the antimicrobial environment of the late phagosome and phagolysosome. It has been demonstrated that in closely related *M. avium*, *in vitro* cytokine treatment of macrophages with TNF-α or IFN-γ increases bacteriostatic and bactericidal capacity of the macrophages(2, 16). The IFN-γ/LPS combination has been shown to enhance inducible nitric oxide (iNOS) activity and to restrict the ability of *M. avium* and *M. bovis* to interfere with phagosomal maturation(1, 17, 19). Interestingly, interference with cytokine activation pathways has been shown to be an

additional mechanism used by pathogenic mycobacteria to promote intracellular survival within macrophages(9).

The results of this study demonstrate that there was increased ability of IFN-γ/LPS treated macrophages to acidify the phagosome containing *M. a. ptb* compared to those in non-treated macrophages (Figure 1). Acidification of the phagosomes containing *M. a. ptb* was evaluated by determining the extent of colocalization of these phagosomes with the acidotrophic fluorigen Lysotracker Red. Lysotracker Red is a highly specific marker of acidic organelles, and overlap of this marker with labeled *M. a. ptb* indicates acidification of the phagosome containing *M. a. ptb*. Consistent with a report in progress by authors of this manuscript, in non-treated macrophages the low level of colocalization of *M. a. ptb* with acidic compartments compared to the high levels for killed *M. a. ptb* and zymosan A suggests that *M. a. ptb* is capable of inhibiting acidification of the phagosome in which it resides and that the bacterial viability is required to inhibit acidification (Table 1, Figure 1)(11). In contrast, in the current study, acidification was similar for phagosomes containing live and killed *M. a. ptb* in IFN-γ/LPS treated macrophages suggesting that treatment of the macrophages with IFN-γ/LPS restores the ability of these macrophages to acidify the phagosome containing *M. a. ptb* (table 1).

These data are consistent with studies that evaluated the effect of cytokines on acidification of phagosomes containing pathogenic mycobacteria. These studies reported increased capacity of macrophages treated with IFN-γ and/or LPS to acidify phagosomes containing mycobacteria. The mechanism leading to increased acidification appears to be enhanced recruitment of the V-ATPase proton pump to the phagosome containing mycobacteria (2, 17, 19). Accumulation of Lysotracker Red within phagosomes has been

shown to be dependent on V-ATPase since its presence within phagosomes can be nearly completely inhibited by addition of the V-ATPase inhibitor Bafilomycin A_1 (19). In the current study increased colocalization of Lysotracker red with *M. a. ptb* in IFN-γ/LPS treated macrophages is consistent with increased V-ATPase activity within the mycobacterial phagosomal as a mechanism leading to phagosomal acidification in cytokine treated macrophages.

There were increased numbers of phagosomes containing *M. a. ptb* which were enriched with Lamp-1 in IFN-γ/LPS treated macrophages compared to non-treated macrophages. This suggests enhanced ability of treated macrophages to cause maturation of phagosome containing *M. a. ptb* into later phagosomal stages. Phagosomal maturation has been shown to be dependent on stepwise fusion and fission events between phagosomes and early and late endocytic compartments, and through these events developing phagosomes transiently acquire proteins characteristic of early then late endocytic compartments(8, 15). In the current study, in non-treated macrophages the numbers of phagosomes containing live *M. a. ptb* enriched with the Lamp-1 marker were reduced from those containing killed *M. a. ptb* (Table 2, Figure 2). Reduced acquisition of Lamp-1 by the phagosome containing *M. a. ptb* in non-treated macrophages suggests decreased interaction of the phagosome containing *M. a. ptb* with late endocytic compartments, and therefore, restricted maturation into late phagosomal stages. These results are similar to those of a report in progress by the authors of this manuscript, which showed increased Lamp-1 accumulation within phagosomes containing killed *M. a. ptb* and zymosan A over those containing live *M. a. ptb*, showing that viability was required for *M. a. ptb* to interfere with Lamp-1 acquisition (12). In the current study the numbers of phagosomes containing the Lamp-1 marker were similar for live and

killed *M. a. ptb* in IFN-γ/LPS treated macrophages (Table 2, Figure 2). These results indicate that in IFN-γ/LPS treated macrophages there is enhanced ability for fusion of the phagosome containing *M. a. ptb* with late stages of the endocytic pathway leading to accumulation of late endosome/lysosome proteins within these phagosomes and subsequent maturation into late phagosome and phagolysosome stages. These results are consistent with a similar study where it was shown that in IFN-γ/LPS treated macrophages there were reduced numbers of *M. avium* phagosomal compartments with positive labeling for the transferrin receptor, an early endosomal marker receptor, indicating decreased ability to sustain interactions with early endosomal compartments (17).

Treatment of macrophages with IFN-γ and LPS led to decreased percent survival of *M. a. ptb* compared to *M. a. ptb* in non-treated macrophages, and *M. smegmatis* in treated and non-treated macrophages (Table 3). Consistent with a report in progress by these authors, the percent survival of *M. a. ptb* in non-treated macrophages remained fairly constant during the infection periods; however, in contrast to that report there were no significant difference in percent survival between *M. a. ptb* and *M. smegmatis* in non-treated macrophages in the current study(12). This may in part be due to variation in the percent of live bacteria in the original inoculum. Although not statistically significant, the values for percent survival of *M. smegmatis* were decreased from *M. a. ptb* from the 24 hour time point on suggesting a trend for decreased survival of *M. smegmatis* in non-treated macrophages compared to *M. a. ptb*. IFN-γ/LPS treatment of macrophages did not effect survival of *M. smegmatis*, which may indicate that the effects of cytokines on macrophages may vary with different infecting species of mycobacteria. In the current study we identified a reversal in the trends for inhibition of phagosomal development at 4 hours in IFN-γ/LPS treated macrophages by

confocal microscopy; however, no significant changes in percent survival of *M. a. ptb* in treated macrophages were detected until the 24 hour time period. This is consistent with a similar report which reported delayed killing of *M. avium* in IFN-γ/LPS treated macrophages despite a detection of maturation of its phagosomal compartment early in the course of infection(17). These results together with those of the current study suggest that while the ability to cause acidification and maturation of the *M. a. ptb* phagosome in IFN-γ/LPS treated macrophages may be an early event, it does not indicate immediate decreases in viability of the organism (17).

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Table 1. Mean percent colocalization of bacteria with Lysotracker Red in IFN-γ/LPS activated macrophages: Confocal microscopy was used to identify at least 50 random bacteria at 4, 24, and 48 hours. The number of yellow (colocalized) bacteria were counted and the results expressed as a percentage of total (colocalized and noncolocalized) bacteria. Each value is the mean percentage of 2 replicates. Values are means of all time points.

Mean % colocalization for all time points		
M. a. ptb	**Non treated macrophages**	**IFN-γ/LPS treated macrophages**
Live	**52.9^{A}**	**75.5^{B}**
Killed	**87.5^{B}**	**86.1^{B}**

Means not sharing the same letter are statistically different (P<0.05).

Table 2. Mean percent colocalization of bacteria with Lamp-1 in IFN-γ/LPS activated macrophages: Confocal microscopy was used to identify at least 50 random bacteria at each time point4, 24, and 48 hours. The number of yellow (colocalized) bacteria were counted and the results expressed as a percentage of total (colocalized and noncolocalized) bacteria. Each value is the mean percentage of 2 replicates. Values are means of all time points.

Mean % colocalization for all time points		
M. a. ptb	**Non treated macrophages**	**IFN-γ/LPS treated macrophages**
Live	**39.0^{A}**	**68.3^{B}**
Killed	**75.6^{B}**	**74.4^{B}**

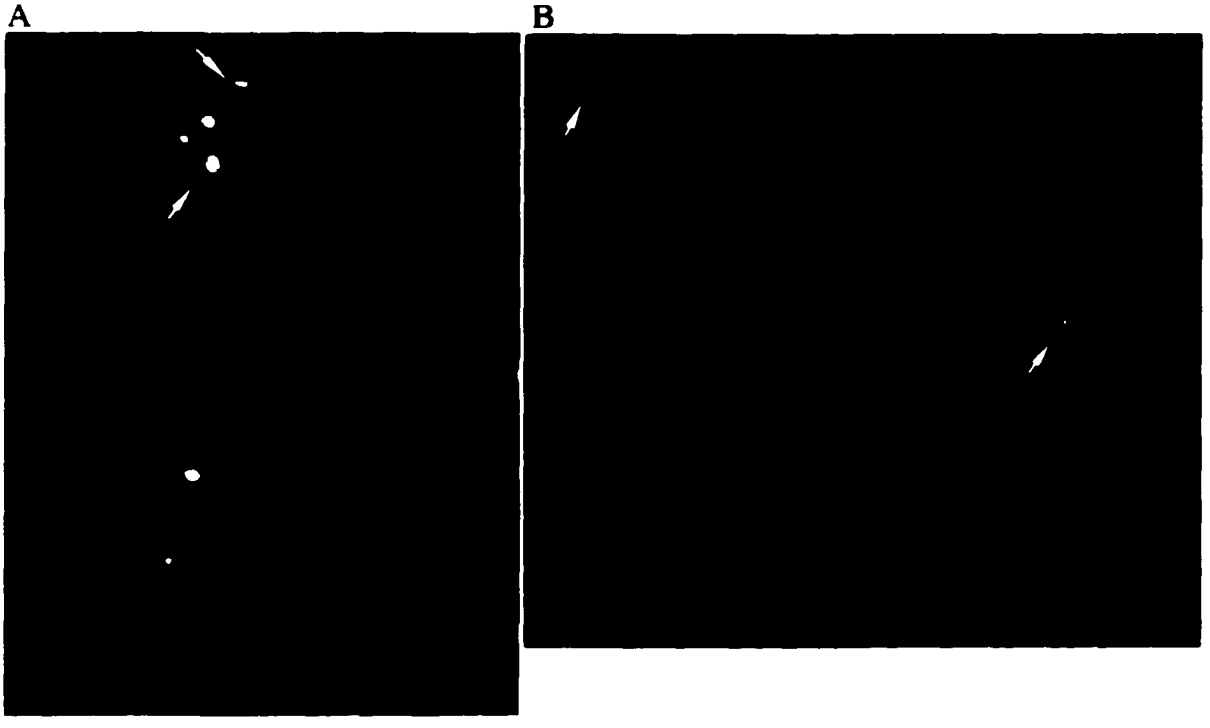
Means not sharing the same letter are statistically different (P<0.05).

Table 3: Percent survival of *M. a. ptb* and *M. smegmatis* in IFN-γ/LPS treated and non-treated macrophages: Macrophages treated with IFN-γ/LPS and non-treated macrophages were infected with either *M. a. ptb* or *M. smegmatis.* Following each time point macrophages were lysed and viability of the released bacteria was determined by the BacLight staining system.

Bacteria	Treatment	Percent Survival			
		4 hour	24 hour	48 hour	72 hour
M. smegmatis	None	76.8^{A}	50.0^{AB}	54.8^{A}	42.0^{A}
M. smegmatis	IFN-γ/LPS	67.8^{A}	43.8^{AB}	60.33^{A}	45.2^{A}
M. a. ptb	None	63.8^{A}	61.4^{A}	62.3^{A}	59.3^{A}
M. a. ptb	IFN-γ/LPS	62.7^{A}	41.8^{B}	39.0^{B}	39.4^{A}

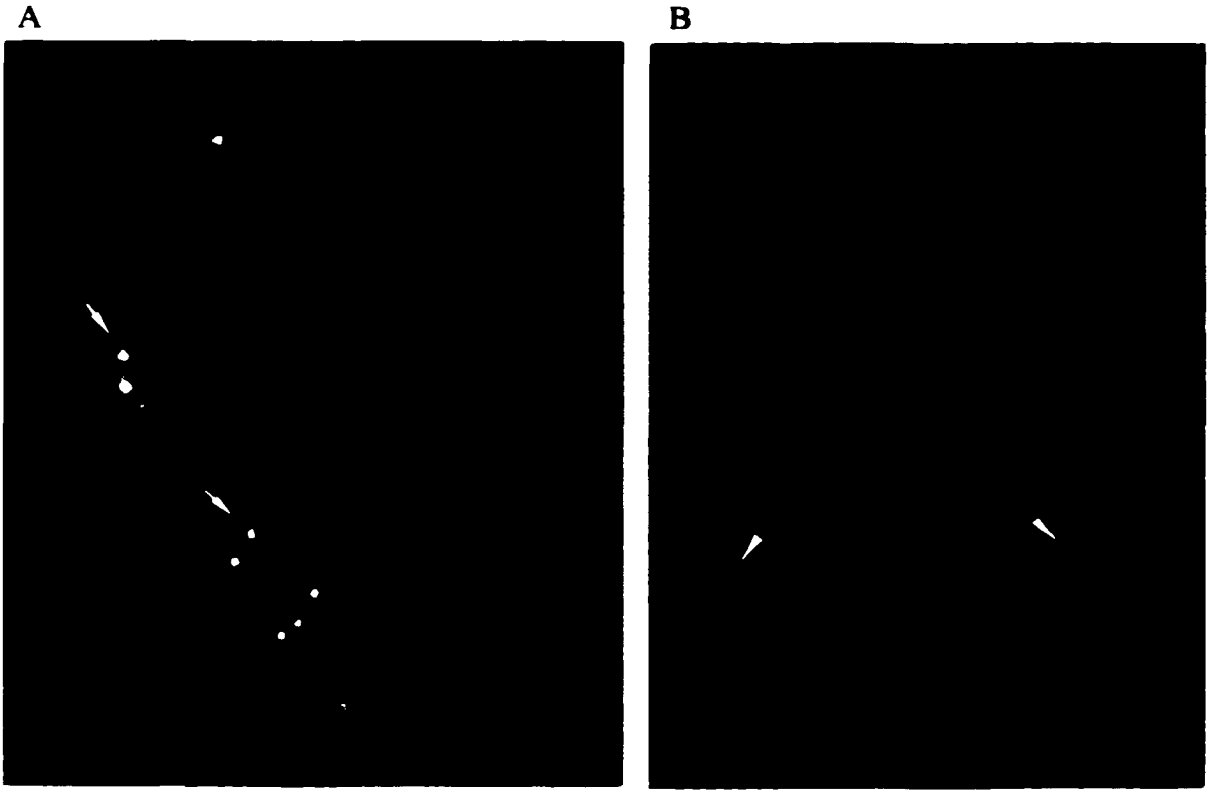
Means (% live) including six replicates. Means within a column not sharing a letter are significantly different (P>.05)

figure 1



Colocalization of *M. a. ptb* with Lysotracker Red labeled compartments in IFN-γ/ LPS treated macrophages. IFN-γ/LPS treated (A) and non-treated (B) macrophages were infected with *M. a. ptb* conjugated to FITC. At each time point acidic organelles were labeled by the addition of Lysotracker Red. In IFN-γ/LPS treated macrophages *M. a. ptb* (arrows) appear colocalized (yellow) indicating that they are within compartments of low pH, which suggests that treated macrophages have increased ability to acidify the *M. a. ptb* phagosome (A). In non-treated macrophages *M. a. ptb* (arrows) appears noncolocalized (green) indicating that these bacteria reside within a compartment that is not acidified, which demonstrates *M. a. ptb* inhibition of phagosomal acidification in non-treated macrophages.

Figure2



Colocalization of *M. a. ptb* with Lamp-1 compartments in IFN-γ/LPS treated macrophages. IFN-γ/LPS treated (A) and non-treated (B) macrophages were infected with *M. a. ptb* conjugated to FITC. Following each time point Lamp-1 was immunofluorescently labeled. In IFN-γ/LPS treated macrophages *M. a. ptb* (arrows) appear colocalized (yellow) indicating that they are within compartments which are enriched with the Lamp-1 marker, which suggests that treated macrophages have increased ability to cause maturation of the *M. a. ptb* phagosome (A). In non-treated macrophages *M. a. ptb* (arrows) appear noncolocalized (green) indicating that they reside within a compartment that has low levels of the Lamp-1 marker, which demonstrates *M. a. ptb* inhibition of phagosomal maturation in non-treated macrophages.

GENERAL CONCLUSIONS

Acidification of the Phagosome Containing *M. a. ptb* is Impaired in J774 Macrophages

In Chapter Two, we tested the hypothesis that *M. a. ptb* resides inside macrophages within a phagosomal compartment that has reduce ability to become acidified. Acidification of the phagosome containing *M. a. ptb* was characterized by determining extent colocalization of Lysotracker Red, a marker of acidic organelles, with FITC labeled *M. a. ptb* by laser confocal microscopy. To further evaluate acidification of phagosomes containing *M. a. ptb*, we evaluated intensity of immunogold labeling of DAMP, a fixable marker of low pH compartments, within the phagosome containing *M. a. ptb* by transmission electron microscopy.

The results of these studies supported the hypothesis that *M. a. ptb* can impair phagosomal acidification. The confocal microscopy studies demonstrated limited colocalization of phagosomes containing *M. a. ptb* with Lysotracker Red, indicating that noncolocalized bacteria were present within phagosomal compartments with restricted ability to acidify. The results from the ultrastructural study supported the colocalization findings studies by demonstrating low levels of immunogold labeling of DAMP within the phagosomes containing *M. a. ptb*, therefore demonstrating reduced acidification phagosomes containing *M. a. ptb*. We demonstrated that following the addition of a Bafilomycin A_1, a specific inhibitor of V-ATPase, Lysotracker Red accumulation within phagosomal compartments containing killed *M. a. ptb* and yeast wall extract (zymosan A) was almost completely inhibited. These results suggest that V-ATPase is required for Lysotracker Red

accumulation, and it is likely that *M. a. ptb* prevents acidification of the surrounding phagosome, and thus Lysotracker Red accumulation, by exclusion of V-ATPase.

Maturation of Phagosomes Containing *M. a. ptb* is Inhibited in J774 Cells

In Chapter Three we tested the hypothesis that *M. a. ptb* survives within macrophages by inhibiting maturation of the phagosomal compartment in which it resides. Maturation of the phagosome containing *M. a. ptb* was evaluated by immunofluorescently labeling two stage specific markers of phagosomal maturation, the TFR and Lamp-1, and determining the extent of colocalization of these markers with FITC labeled bacteria by laser confocal microscopy.

The results of colocalization studies supported the hypothesis of restricted maturation of the phagosome containing *M. a. ptb*. We demonstrated that increased numbers of phagosomes containing *M. a. ptb* colocalized with the TFR, a marker of early endosomal compartments, compared to phagosomes of yeast cell wall extract zymosan A. These data suggest that colocalized *M. a. ptb* reside within phagosomes that are enriched with the TFR. Reduced numbers of phagosomes containing *M. a. ptb* colocalized with the late endosome/lysosomal marker Lamp-1 compared to phagosomes containing zymosan A, indicating that noncolocalized *M. a. ptb* resides within a phagosomal compartment that has low levels of Lamp-1. Taken together, increased TFR and decreased Lamp-1 within the phagosomal membrane surrounding *M. a. ptb* suggest inhibited phagosomal maturation by showing that these phagosomes have retained characteristics of early phagosomes/endosomes and have not acquired markers associated with late stages of the phagosomal pathway.

M. a. ptb Needs to be Viable to Inhibit Phagosomal Development

In Chapter Two and Chapter Three we evaluated the effect of bacterial viability on the ability of *M. a. ptb* to interfere with normal development of its phagosomal compartment. We accomplished this by comparing colocalization of phagosomal markers for acidification and maturation in live and killed *M. a. ptb*. In addition to colocalization studies, in Chapter Two, acidification of phagosomes containing live and killed *M. a. ptb* were evaluated by assessing intensity of immunogold labeling of DAMP within these phagosomes by transmission electron microscopy.

The results of these studies indicate that *M. a. ptb* needs to be viable in order to inhibit phagosomal acidification and maturation. Colocalization of killed *M. a. ptb* with Lysotracker Red was markedly increased over that of live *M. a. ptb*, indicating increased acidification of the phagosomes containing killed *M. a. ptb*. Following killing of *M. a. ptb* there was a reversal of the trends characterized in Chapter Three for colocalization of live *M. a. ptb* with markers of phagosomal maturation. Colocalization of killed *M. a. ptb* with the TFR was markedly reduced from that of live *M. a. ptb*, which indicates loss of TFR from the phagosomal membrane surrounding killed *M. a. ptb*. There were increased numbers of phagosomes containing killed *M. a. ptb* which colocalized with Lamp-1 compared to phagosomes containing live *M. a. ptb*, indicating enrichment of these phagosomes with the Lamp-1 marker. These results support normal phagosomal development of the phagosome containing killed *M. a. ptb* by demonstrating that these become acidified, lose markers of early phagosome/endosomes, and acquire those of late phagosome/phagolysosomal stages. This is further supported by data showing that killed *M. a. ptb* had a similar extent of colocalization with markers of phagosomal development as did the control agent zymosan A.

In addition to colocalization studies, transmission electron microscopy evaluation of DAMP accumulation demonstrated increased acidification of phagosomes containing killed *M. a. ptb* by showing increased intensity of labeled DAMP within phagosomes containing killed *M. a. ptb* compared to those containing live *M. a. ptb*

IFN-/LPS Treatment of J774 Cells Leads to Increased Acidification and Maturation of the *M. a. ptb* Phagosome

The results detailed in Chapter Four support the hypothesis that IFN-γ /LPS treatment of macrophages leads to increased acidification and maturation of the phagosome containing *M. a. ptb*. This hypothesis was tested by infection of non-treated and IFN-γ /LPS treated macrophages with *M. a. ptb*, and characterization of colocalization of *M. a. ptb* with markers of phagosomal acidification and maturation. The results showed that in IFN-γ /LPS treated macrophages there was increased colocalization of *M. a. ptb* with Lysotracker Red compared to that of non-treated macrophages, which indicates enhanced ability of treated macrophages to acidify the phagosome containing *M. a. ptb*. In addition there was increased colocalization of *M. a. ptb* with Lamp-1 in IFN-γ /LPS treated macrophages compared to non-treated macrophages. This indicates that the phagosomes containing *M. a. ptb* are enriched with Lamp-1 in IFN-γ /LPS treated macrophages. Taken together these results demonstrated that following treatment of macrophages with IFN-γ /LPS, there was a shift in *M. a. ptb* phagosomal characteristics away from that of an early phagosomal compartment towards a late phagosome/phagolysosomal stages.

In Chapters Two and Three phagosomal compartments containing killed *M. a. ptb* were found to have similar extents of acidification and maturation as the control agent zymosan A. The extent of colocalization of live *M. a. ptb* with Lysotracker Red and Lamp-1 in treated macrophages was comparable to that of killed bacteria suggesting similar fates of phagosomes containing live and killed *M. a. ptb* in IFN-γ /LPS treated macrophages.

There are Increased Percentages of *M. a. ptb* which Survive within J774 cells Compared to *M. smegmatis*; however, Percent Survival of *M. a. ptb* is Reduced from *M. smegmatis* in IFN-γ /LPS Treated Macrophages

In Chapter Two we demonstrated that there were increased percentages of *M. a. ptb* surviving within J774 cells compared to the nonpathogenic species *M. smegmatis*. This was accomplished by infecting J774 cells with either *M. a. ptb* or *M. smegmatis* and following infection periods lysing cells and staining the recovered bacteria for viability with the Bac*Light* Live/Dead kit, which differentiates live from dead bacteria. The data from this study demonstrated that there was a significant decrease in percentages of live *M. smegmatis* compared to *M. a. ptb* following a 24 hours infection period. In Chapter Four we demonstrated that in macrophages treated with IFN-γ /LPS there was a decrease in percent survival of *M. a. ptb* compared to percent survival of *M. a. ptb* in non-treated macrophages. Additionally the percent survival of *M. a. ptb* in IFN-γ /LPS was reduced from that *of M. smegmatis* in both IFN-γ /LPS treated and non-treated macrophages. The reasons for differences in cytokine effects on intracellular survival of *M. smegmatis* and *M. a. ptb* are unknown. It may be that cytokine effects on intracellular killing of macrophages may vary

with species of mycobacteria. Intracellular killing of nonpathogenic *M. smegmatis* may be at its maximum in non treated macrophages and IFN-γ/LPS treatment will not raise this level. In contrast IFN-γ/LPS treatment may allow the macrophage to overcome the *M. a. ptb* ability to resist intracellular killing leading to reduced intracellular survival following treatment of macrophages.

Future Directions

In the future we would like to use this *in vitro* system to evaluate gene deletion mutants of *M. a. ptb*, potentially identifying candidates for further *in vivo* testing and vaccine development. We are also interested in evaluating additional parameters in *M. a. ptb* infected J774 cells including changes in calcium concentrations following uptake of *M. a. ptb*, and the effect of platelet activating factor on intracellular survival of *M. a. ptb*. Our *in vitro* system is based on a murine cell line and it is our hope to begin evaluation of the parameters described in this dissertation in bovine macrophages. At this time we are limited in our ability to do immunofluorescent staining of phagosomal markers due to a lack of antibody against bovine phagosomal markers; however we are at the point of being able to evaluate acidification of the phagosome containing *M. a. ptb* within bovine macrophages.

APPENDIX: GENERAL OVERVIEW OF PARATUBERCULSOSIS

Paratuberculosis

Paratuberculosis is an economically important contagious disease of ruminants with worldwide distribution. Johne and Frothingham first described the disease in 1895, and in 1910 Twort and Ingram identified a mycobacterium as the cause. (4). The agent has had different names since it's identification including *Mycobacteria enteriditis chronicae pseudotuberculosae bovis johne*, *Mycobacteria johnei* and more recently *Mycobacterium paratuberculosis*. Today the organism is classified as a subspecies of *Mycobacterium avium* and is named *Mycobacterium avium* subspecies *paratuberculosis* (*M. a. ptb)*(14, 15). Paratuberculosis is common in domestic ruminants including dairy and beef cattle, sheep, and goats, and also occurs in free ranging ruminants such as camels, antelope, bison, llamas, and mountain goats (6). Occasionally disease is reported in pigs, horses and wild rabbits (1). Experimental infection has been established in a several species including rats, mice, and gerbils (2). Economic losses are substantial and mainly are due to decreased production and culling of affected animals from the herd. In 1989 the annual cost to the livestock industry in the USA was estimated to be 1.5 billion dollars per year (16). There is also speculation that *M. a. ptb*. plays a role in the development of Crohn's disease, a progressive and debilitating inflammatory bowel disease of humans (25, 26, 28).

Paratuberculosis is a chronic enteric disease characterized by diarrhea, progressive weight loss, and poor response to medical intervention. In cattle there is pronounced weight loss combined with effortless watery diarrhea. Frequently diarrhea waxes and wanes with long intermittent periods of remission. In sheep and goats diarrhea is a less consistent feature;

however, wasting and poor coat condition are typical(1, 11). Most animals continue to eat and are alert; however, as the disease progresses, there is decreased milk production, infertility, anemia, edema, cachexia, and eventually death. Treatment of paratuberculosis is usually ineffective. Antibiotics used in the treatment of tuberculosis such as clofazimine, isoniazid, rifampin, and streptomycin have been tested for use in paratuberculosis with little effect (24). Even if clinical signs improve the animals continue to shed bacteria into the environment, and due to expense and long period of time required, medical treatment typically is not pursued(6, 25).

Initial infection usually occurs in very young animals; however, clinical disease is not apparent until adulthood. Animals less than 30 days of age are most susceptible likely due to the immaturity of their immune systems (4). Animals greater than 6 months tend to be resistant to infection (4). The major route of transmission is ingestion of bacteria by feeding on contaminated pastures. It is estimated that infected animals can pass 10^8 bacteria per gram of feces with as many as 10^{12} bacteria shed in feces daily (3). This leads to heavy contamination of the environment and facilitates spread to young animals throughout the herd. Infected mothers will shed bacteria into the colostrum with subsequent transmission to neonates (3). Additionally, intrauterine spread to the fetus has been incriminated in females in the advanced stages of disease (6).

The incubation period of Johne's disease is long and variable. Typically clinical signs do not usually develop until 2-5 years of age, and are often associated with some kind of stress such as parturition, dietary deficiency, or concurrent disease (1). The clinical disease has been divided into three stages (6). The first includes animals that have been infected but are not showing clinical signs and are not shedding bacteria. In the second stage animals

remain asymptomatic but are shedding bacteria in feces. Finally in the third stage animals are in the clinical stages of disease and are shedding bacteria. Not all infected animals progress to clinical disease; however, many of these subclinical animals will shed bacteria and contribute to contamination of the environment. It is speculated that there are more asymptomatic carriers than animals with overt disease (6).

Consistent with other mycobacterial diseases, paratuberculosis is characterized by granulomatous inflammation. In cattle lesions are typically present in the intestine and mesenteric lymph nodes. Grossly the intestinal wall is thickened and the mucosal surface has an irregular corrugated appearance. Lesions are most common near the terminal ileum, but may be present diffusely or segmentally from duodenum to anus (1). The lymphatic vessels within the affected intestine and associated mesentery are typically dilated and prominent due to lymphangitis, and mesenteric lymph nodes are markedly enlarged. Microscopically, the lesion in the intestine is characterized by expansion of the lamina propria by large numbers of macrophages and occasional multinucleate giant cells with atrophy and fusion of the villi and hyperplasia of the crypt epithelium. There is often variability in the amount granulomatous infiltrate, especially early in the course of disease, and infiltrates of lymphocytes, plasma cells and eosinophils are also a common microscopic finding. A feature that is unique to bovine paratuberculosis, and distinguishes it from the intestinal form of tuberculosis, is the lack of caseous necrosis within the granulomatous inflammation (1).

The lesions in sheep and goats are similar to those in cattle with few exceptions. Lesions are present within the intestinal tract and mesenteric lymph nodes, but are also common in lymph nodes throughout the body. Unlike cattle, the granulomatous lesions may contain foci of caseous necrosis, making differentiation from tuberculosis more difficult (1).

In most species the severity of lesions is variable from case to case and often there is a lack of correlation between clinical signs and the severity of the gross and microscopic lesions.

Clinical signs are the result of protein loss into the intestinal lumen. Atrophy of the intestinal villi lead to a reduction in the absorptive surface area with subsequent malabsorption and maldigestion of amino acids. As the granulomatous inflammatory infiltrate develops within the lamina propria, there is further loss of protein through increased fluid secretion into the intestinal lumen, which overwhelms the large intestine's reabsorptive capacity (11). Hypoproteinemia develops with continued losses and leads to weight loss from a negative nitrogen balance and edema from decreased plasma oncotic pressure.

The lesions of paratuberculosis tend to be localized; however, the infection is systemic. In the intestine, the organisms are taken up by M cells in the ileal dome and then passed to subepithelial macrophages of the Peyer's patches (19). Bacteria are then phagocytized where they reside intracellularly and begin to replicate. As the organisms replicate, peripheral monocytes are attracted to the area, activated and granulomatous inflammation develops(6, 32). The organisms subsequently enter lymphatics, are carried to the mesenteric lymph nodes, and eventually throughout the body. Following experimental infection bacteria can be isolated from the mesenteric, suprapharyngeal lymph nodes, and tonsil at 1-2 months post infection (17). In both clinical and asymptomatic animals *M. a. ptb* can be isolated from multiple organs, blood, urine, and milk (1).

The Genus Mycobacterium

Mycobacteria are aerobic, acid fast, non-motile and non-spore forming bacteria with slow growth rates and a wide host range. They range in size from .2 to .6 by 1 to 10 μm in

size, and may form filaments which easily fragment into rod or coccoid bacteria(11, 20). The cell walls of all species contain a large amount of lipid including waxes containing mycolic acids with long branched chains. The high lipid content is responsible for the acid-fast staining characteristic. Mycobacteria do not stain well with the gram stain, but are considered to be gram positive(11, 13, 20). Mycobacteria are typically slow growing and, although times vary with different species, take up to 8 weeks or more following culture for visible colonies to appear (13). Those species that cause disease infect a wide range of hosts including numerous domestic and exotic species, however the susceptibility to a given species of mycobacteria varies with different animal species (13).

A number of systems have been developed to classify species of mycobacteria. A common system divides species into three categories: those that are pathogenic, those that are nonpathogenic or opportunistic, and the saprophytes which are typically environmental organisms (13). Typically, species are also divided into those that cause tuberculous disease characterized by multiple discrete granulomas with caseonecrotic cores, and those that do not. The species that cause tuberculosis are *M. tuberculosis*, *M. bovis*, and *M. avium*. The term "Mycobacteriosis" is given to disease that is caused by the remainder of mycobacterial species (1). Several systems are described for the mycobacteria that do not cause tuberculous disease. The Runyon scheme separates species by colony morphology, pigmentation, and growth rate (22). A system described by Wolinsky, and modified by Woods and Washington groups non-tuberculous mycobacteria by their pathogenicity to humans(29, 30). Rate of growth is also important to the classification of mycobacteria, and three categories based on growth rate are described. First, are the fast growing species that form colonies within one week or less. Second are intermediate species with 1-2 weeks for colony formation. The third

category includes species that take greater than 2 weeks to grow. Within this category are species such as *M. a. ptb* with extremely slow growth rates that may take up to 16 weeks for visible colonies to appear(11, 13). Generally, mycobacteria that have rapid growth rates tend to be nonpathogenic to humans and animals, while the slow growers are associated with disease; however, there are notable exceptions (23).

Paratuberculosis is caused by a mycobactin – dependent species that is classified as a subspecies of *Mycobacterium avium*. Mycobactins are complex lipids that chelate iron, and are present in the wall of Mycobacteria and Nocardia (6). For certain species of mycobacteria, mycobactin is an essential factor required for growth in media and such species are called the mycobactin-dependent mycobacteria. Some however will lose their requirement for mycobactin as they are serially passaged in media (14). In addition to *M. a. ptb*, mycobacteria isolated from wood pigeons and free ranging deer are also mycobactin – dependent (18).

M. a. ptb was initially given separate species status and was named *Mycobacterium paratuberculosis*. Immunodiffusion analysis showed strong antigenic similarities between *Mycobacterium paratuberculosis*, as well as other mycobactin-dependent mycobacteria, with *Mycobacterium avium* (18). Genetic analysis has demonstrated >90% homology between the genomes of the *Mycobacterium paratuberculosis* and *Mycobacterium avium* leading to reclassification of *Mycobacterium paratuberculosis* as a subspecies of *Mycobacterium avium*(14, 15, 27). In addition to its taxonomic classification *M. a. ptb* is also characterized by the classification systems described previously as a non-tuberculous, slow growing, pathogenic mycobacterium that belongs to the Runyon group IV.

M. a. ptb, like other pathogenic mycobacteria, is an intracellular pathogen of mononuclear phagocytes; however, the mechanisms by which it survives within the hostile environment of the macrophage are not entirely understood. Macrophages kill and digest phagocytosed organisms through generation of several toxic substances including oxygen derived free radicles, nitric oxide, acid hydrolases, and proteolytic enzymes (7). Determining how intracellular pathogens subvert these destructive processes is an area of research interest. Numerous advances have been made using cultured macrophages to evaluate intracellular survival of several species of mycobacteria including *Mycobacterium tuberculosis* and *Mycobacterium avium* (5, 9). These and other studies have demonstrated that pathogenic mycobacteria reside within an intracellular vesicle (phagosome) that avoids fusion with lysosomes(10, 12). Within this environment the bacteria are protected from degradation and are able to survive and replicate. Intracellular trafficking of mycobacterial phagosomes and properties that prevent their fusion with lysosomes are current areas of research interest(5, 8, 9, 21, 31).

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